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# CHEMICAL STUDIES ON BACTERIAL AGGLUTINATION

## V AGGLUTININ AND PRECIPITIN CONTENT OF ANTISERA TO HAEMOPHILUS INFLUENZAE, TYPE B\*

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Agglutinating and precipitating antisera to *Haemophilus influenzae*, type B, have been recorded in recent years by Pittman (1), and by Ward and Wright (2), and have also been studied by Pittman and Goodner (3), and Pothergill, Dingle, and Chandler (4). The titers of these sera were determined by the usual relative methods. Knowledge of the actual antibody content of anti influenza type B sera has now become urgent because of indications that such sera may possess curative action in influenzal meningitis in children (5). The quantitative, absolute methods for the estimation of agglutinin nitrogen (6) and precipitin nitrogen (7) developed in this laboratory proved adaptable to the problem. The present paper deals with the agglutinin content of antisera obtained from the horse and rabbit as estimated with suspensions of influenza bacilli prepared in different ways and with different strains, and also discusses the precipitin content of such sera as determined with different preparations of the specific polysaccharide of the type B influenza bacillus. The methods used for obtaining antisera from rabbits are also described, since the analytical control has aided in the production of rabbit sera containing five to ten times as much antibody as the best available when this study was initiated.

*1 Preparation of Haemophilus influenzae Type B Suspensions and Immunization of Rabbits*—The fundamental principles stressed in a previous report (5) have been adhered to in the production of the antigen used for immunization. Well encapsulated type B strains recently isolated from blood or spinal fluid cultures have been used. When the ability of these strains to produce well defined capsules diminished on transplanting, a mucin suspension of the culture was passed through mice. The first transplant after recovery from the peritoneum was used for seeding Levinthal (8) agar plates. 0.3 ml of a 24 hour Levinthal broth culture was spread over each plate and incubated for 6

\* The work reported in this communication was carried out in part under the Harkness Research Fund of the Presbyterian Hospital.

hours The growth was then washed off with 5 ml of 0.85 per cent sodium chloride containing 0.5 per cent of formalin. The resulting suspension was diluted to a standard turbidity of 3.5 by the Gates turbidometer. This type of antigen may be stored in the cold for at least a month and still retains definite capsules when tested by the swelling phenomenon (5). Since there is evidence that the type specific cells are very susceptible to temperature changes and mechanical influences, the stored antigen was checked frequently for presence of capsule. This standard suspension, averaging 1,800,000,000 per ml by colony counts, was used according to the following schedule in the production of rabbit antisera. Immediately before injection the antigen was diluted with an equal quantity of saline. 24 New Zealand white male rabbits about 6 months of age were divided into 2 groups of 12 each.

TABLE I  
*Schedule of Injections of Rabbits with Haemophilus influenzae, Type B*

Rabbit group 1-12	Vaccine			Agglutinin nitrogen in serum pool at end of course (from Table IV)
	Daily dose 4 times weekly	Number of injections	Total quantity	
	ml		ml	mg per ml
Course I	0.1-1.0	16	10.1	0.60
Course II	1.0	14	14.0	0.60
Course III	0.1-1.0	13	10.7	0.83
Course IV	0.5-1.0	13	12.0	1.08
Total			46.8	
13-24				
Course I	0.1-1.0	16	10.1	0.50
Course II	1.0-3.0	14	24.4	0.77
Course III	0.1-3.0	13	21.2	0.77
Course IV	0.5-3.0	13	28.0	0.73
Total			83.7	

During the first month of immunization rabbits 1 to 12 received the standard vaccine and rabbits 13 to 24 the standard vaccine washed once with 0.5 per cent formalinized saline. The same schedule and dosage were used for each group. With an initial dose of 0.1 ml and with inoculations on 4 consecutive days out of each week the amount was increased by 0.1 ml daily until 1.0 ml was reached. This dose was repeated for the next 5 injections, after which a rest period of 6 days elapsed prior to sample bleedings.

Since analysis of these failed to show much difference between the antisera produced by the washed and unwashed vaccines (Tables I and IV), unwashed vaccine was used for all subsequent immunizations. In an attempt to determine the optimum dosage of antigen rabbits 1 to 12 were injected with a constant amount, 1.0 ml, and rabbits 13 to 23<sup>1</sup> were increased gradually to 3 ml. After large bleedings of 40 ml per rabbit, 0.1 ml

<sup>1</sup> During the first four courses of injections 3 rabbits died in each group

of antigen was given the following day. The schedule used during the previous course was then resumed for each group of rabbits for an additional 2 months with 40 ml bleedings per rabbit at monthly intervals. The analysis of the antibody content at these intervals showed that while the group 13 to 23, receiving up to 3 ml of antigen daily, showed a more rapid rise, it became stationary sooner and never reached as high a peak. Although this may have been due to individual variations in the two groups, the dose per day for all rabbits was changed to 1.0 ml after a 3 months period of comparison of the dosages described. The immunization procedure is summarized in Table I and the data on the agglutinin content of the pooled bleedings from each series of rabbits are given in Table IV.

Suspensions for the quantitative agglutinin method were prepared in the same way as the vaccines for injection. The formalinized suspensions were allowed to stand in the cold for at least 48 hours prior to centrifugation and removal of the formaldehyde by three washings with chilled 0.9 per cent saline. The cells were then taken up in chilled saline containing 1:10,000 merthiolate<sup>2</sup> and were evenly suspended and filtered through a loose cotton plug in order to remove any lumps not visible to the naked eye. The suspensions were finally adjusted to a volume such that 1 ml contained 0.4 to 0.6 mg of nitrogen.

In several instances cells from broth cultures were used as noted in the tables.

**2. Determination of Agglutinin Nitrogen.**—The method originally used for the estimation of agglutinin nitrogen in antipneumococcus sera (6) was found applicable with little modification other than the neutralization of the antisera to about pH 7 (phenol red) in order to avoid extraction of nitrogen from the bacillary suspension during the analysis. This precaution contributed greatly to the accuracy and reproducibility of the results. The method depends upon the addition of an accurately measured amount of a washed bacterial suspension to an accurately measured volume of antiserum, the relative amounts being so chosen that the bacteria are in excess. After agglutination is complete the difference in nitrogen between the agglutinated bacilli, suitably washed, and the nitrogen content of the same volume of unagglutinated bacilli, gives in milligrams, the agglutinin nitrogen content of the volume of serum chosen. An analysis of the supernatant is also carried out in order to make certain that all antibody has been removed. Details of the method are given in (6). In its application to neutralized anti-influenza sera and plasmas it was found advisable when maximum values for type specific agglutinin were desired, to use formalin killed suspensions of a 6 hour growth of *H. influenzae*, type B. As noted in Table II, there seemed little difference in the rabbit antisera tested whether the bacilli were grown on agar or in broth. Heat killed cultures, however, were found to have lost much capsular substance, so that repeated absorptions with suspensions prepared from them were necessary to remove an amount of agglutinin comparable with that taken out by a single absorption with formalin killed cells.

Usually 1.5 ml of bacillary suspension containing 0.6 to 0.9 mg of nitrogen was added to 1.0 ml of a weak serum or to 1.0 ml of a suitable dilution of a stronger serum. In measuring the suspension into the blank tubes and the sera great care must be taken to exclude bubbles from the pipette. If this is done a Krogh pipette (9) may be used.

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Using a refrigerating centrifuge manufactured by the International Equipment Co. Boston.

<sup>2</sup> Manufactured by Eli Lilly and Sons, Indianapolis.

to advantage, particularly when many analyses are to be run. This pipette may be set at the desired volume which need not even be accurately known, since the pipette delivers exactly the same volume into the blank tubes as into the analyses. Much labor and eye strain may be avoided in this way.

TABLE II

*Agglutinin Nitrogen Carried Down from Anti-Influenza Type B Sera by Haemophilus influenzae Suspensions*

Horse sera	Dilution	Volume of dilution used	Influenza bacillus suspension	N content of control suspension	N content of agglutinated suspension	Difference (agglutinin N)	Agglutinin N in supernatant on 2nd absorption	Total agglutinin N	Agglutinin N per ml of undiluted serum
		ml		mg	mg	mg	mg	mg	mg
I 60	1 5	1 0	Type B, FK	0 760	0 898	0 138	0	0 14	0 70
"	"	1 0	Unclassified, MK	0 714	0 736	0 022	0	0 02	0 10
712	1 1	1 0	" "	0 714	0 808	0 094	0 003	0 10	0 20
"	"	0 50	Type B, FK	0 874	0 944	0 070	0 014	0 08	0 32
Rabbit sera									
Pool a*	Undil	1 0	" " "	0 878	1 004	0 126	0 012	0 14	0 14
" "	"	1 0	" " " †	0 642	0 742	0 100	0 008	0 11	0 11
13-23 <sub>1</sub>	"	0 5	" " "	0 866	1 120	0 254	0	0 25	0 50
" "	"	0 5	" " " †	0 642	0 874	0 232	0 010	0 24	0 48
13-23 <sub>3</sub>	3 10	1 0	" " HK	0 552	0 652	0 100	0 094 ‡	> 0 19	> 0 63
" "	"	1 0	" " FK	0 606	0 818	0 212	0 020	0 23	0 77
13-23 <sub>4</sub>	"	1 0	" " "	0 760	0 964	0 204	0 018	0 22	0 73
" "	Undil	1 0	R strain, "	0 760	0 818	0 058	0 026	0 08	0 08 §

FK, HK, MK, denote organisms from plate cultures, killed by 0.5 per cent formalin, by heat, by 1:15,000 merthiolate, respectively.

Subnumerals after rabbit serum pool numbers indicate course numbers.

\* Best commercial rabbit serum pool available at beginning of work.

† From broth culture.

‡ 2nd and 3rd absorptions, incomplete.

§ The supernatants from the R agglutinin estimation yielded 0.56 mg per ml type specific precipitin nitrogen.

In the case of an unknown serum preliminary tests may be made rapidly to establish the maximum amount that may be safely used with a given suspension. If an appreciable amount of agglutinin is present the bacilli, if not used in excessive quantity, clump rapidly, even at 0°, and the mixture may be centrifuged and the supernatant tested with more bacilli. Most of the analyses recorded in the tables were carried out at 0°, with

thorough mixing, and were allowed to stand in the ice box until the next day, when the tubes were centrifuged in the cold, carefully decanted as described in (6) and washed twice with chilled saline. In general the supernatants from duplicates were combined before addition of a second measured charge of bacilli since the amount of nitrogen removed in this case was usually nil or very small and much labor could be saved in this way. Indeed, unless the greatest accuracy is desired, analysis of the supernatants need not be completed if, after 48 hours in the cold with occasional mixing, the tubes show no greater sedimentation than the controls. Not more than a few hundredths of a milligram of antibody nitrogen can be present in the supernatant without causing visible agglutination (*cf.* column 8, Table II).

Especially analyzing supernatants it was often impossible to decant from the centrifuged bacilli without loss into the new supernatants and it was then necessary to centrifuge these separately and wash the small amount of sedimented bacilli with the supernatants from the first and second washings of the main portions, after which the two portions of bacilli could be combined. The blanks containing only bacilli and saline, could seldom be decanted without loss even though the last few drops of liquid were left in the tubes, and all supernatants and washings were therefore centrifuged again. The quantitative agglutinin method proved applicable to rabbit plasmas as well as to sera.

In Table II are given analyses of two different horse sera with a type B strain and an unclassified non specific strain of influenza bacillus. Serum I 60 prepared by the Massachusetts State Serum Laboratories and obtained through the courtesy of Dr Leroy D. Fothergill of the Harvard University Medical School contained mostly type specific antibody while serum 712, prepared from old laboratory strains by the New York City Department of Health, contained about one half as much anti type B agglutinin and twice as much non specific agglutinin. The remaining analyses in the table illustrate the behavior of various pools of antisera from rabbits, one of which represents the best available commercially at the inception of this work. As these studies progressed it was found possible to equal in rabbit sera the agglutinin content of the better of the two horse sera, and even higher titers were obtained in later bleedings, as will be noted below. Results with an R strain are given in the last line of the table, showing that relatively little of the antibody in the serum pool tested was non specific.

*3 Preparation of Crude Specific Polysaccharide of Type B Influenza Bacillus*—The existence of a type specific polysaccharide was noted by Pittman (1) and Dingle and Fothergill (10) have recently prepared the carbohydrate from peptone free casein hydrolysate cultures and described its properties. We are greatly indebted to Dr Dingle for a sample of this product. Since three times as much of our own polysaccharide prepared from agar washings of type B culture, was required to precipitate a given amount of antibody N it is concluded that our product consisted of at least two thirds soluble agar derivatives. We shall therefore omit a detailed description of the various batches and give only the method of preparation, since the crude polysaccharide may be obtained fairly easily and is a convenient reagent for the estimation of type specific precipitin in both horse and rabbit anti influenza B sera.

8 to 12 hour growths of type B influenza bacillus on Levinthal agar plates were washed off with the minimum amount of saline and killed with 1 per cent phenol or 0.5 per cent formaldehyde. After several days at room temperature the mixture was centrifuged and the yellowish supernatant treated with acetic acid to maximum turbidity. The

resulting precipitate was centrifuged off in the cold, after which about 10 gm of sodium acetate per 100 ml was dissolved in the supernatant. The solution was treated with 5 to 10 volumes of alcohol and allowed to stand until the resulting fine precipitate had settled. The sedimented material was packed in a centrifuge tube, washed with acetone, and dried *in vacuo*. When the residue was taken up in water considerable insoluble material remained, and this was centrifuged off. The solution was reprecipitated and dried as above until little but water-soluble material remained. At this point one or two shakings with chloroform and butyl alcohol (11) proved helpful in case the biuret reaction was still positive. The water-soluble fraction was taken up in the minimum amount of

TABLE III

*Anticarbhydrate (Precipitin) Nitrogen of Anti-Influenza Type B Sera*

Horse sera	Volume used	Polysaccharide	Amount used	Antibody N precipitated	Antibody N precipitated per ml of serum	Precipitin N Agglutinin N
	ml		mg	mg	mg	per cent
I 60	2 0 (1 5)	Type B, broth	0 5*	0 30	0 75	107
"	" "	" " agar	0 5	0 39	0 98	140
"	" "	Agar (1 1000)	0 3	0 15†	0 38	54
I 60, agar supernatant		Type B, agar	0 5	0 24	0 60	86
<hr/>						
Rabbit sera						
13-23 <sub>1</sub>	1 0	" " "	0 5	0 33	0 33	66
" "	1 0	" " broth	0 15	0 26	0 26	52
13-23 <sub>3</sub>	2 0 (3 10)	" " agar	0 5	0 34	0 57	74
13-23 <sub>4</sub>	0 5	" " broth	0 05	0 30	0 60	82
" " supernatant from R agglutinations		" " "	0 18	1 12	0 56	77
1-11 <sub>4</sub>	0 5	" " "	0 065	0 53	1 06	98

Subnumerals indicate course numbers

\* Unnecessarily large excess

† Deducting blank for N precipitated under similar conditions from non-bacillary horse serum

water and precipitated twice with glacial acetic acid. This removed the phosphate which usually accompanied the polysaccharide up to this point. After reprecipitation with redistilled alcohol and washing with redistilled acetone the product was dried *in vacuo*. Stock solutions of 1 mg per ml were made up for the analyses.

4 *Quantitative Estimation of Type Specific Anticarbhydrate Precipitin in Type B Anti-Influenza Bacillus Sera*—Analyses for anticarbhydrate precipitin were made according to (7) by addition of a slight excess of polysaccharide solution to an accurately measured volume of diluted serum and estimation of the amount of nitrogen in the washed precipitate after 48 hours at 0°C. In the case of the horse sera tested a considerable excess of polysaccharide could be used without causing inhibition of the precipitin

reaction, but the rabbit sera were much more sensitive, especially when the purer carbohydrate from the broth cultures was used. It was therefore necessary to run preliminary experiments on 0.5 ml portions of rabbit serum to determine the amount of polysaccharide necessary to leave a small excess. As precipitation in all but the weakest sera was quite rapid at room temperature these tests consumed little time and little serum. The quantitative precipitin method could be used for rabbit plasmas as well as for sera.

The data obtained are summarized in Table III. It will be noted that in the rabbit antisera the broth culture polysaccharide precipitated nearly as much antibody as did the agar product, and in one instance (Table III, 1-11<sub>4</sub>) the precipitin content equaled the agglutinin content (*cf* Table II). In the other sera the larger agglutinin content is presumably due to antiprotein which would also be removed by the bacilli. The horse sera, however, showed an entirely different behavior, and this is illustrated by the data on serum I 60 in the tables. It is seen that the polysaccharide from the broth culture

TABLE IV

*Agglutinin Nitrogen Content of Sera of Rabbits after Successive Courses of Haemophilus influenzae Type B Injections*

Rabbit sera	Dilution	Volume of dilution used	N content of control suspension	N content of agglutinated suspension	Difference (agglutinin N)	Agglutinin N in supernatant on 2nd absorption	Total agglutinin N	Agglutinin N per ml. of undiluted serum
		ml	mg	mg	mg	mg	mg	mg
1-12 <sub>1</sub>	Undil	0.5	0.866	1.158	0.292	0.006	0.30	0.60
1-11 <sub>2</sub>	3/10	1.0	0.690	0.856	0.166	0.013	0.18	0.60
1-11 <sub>3</sub>		1.0	0.606	0.838	0.232	0.014	0.25	0.83
1-11 <sub>4</sub>	1/3	0.5	0.760	0.932	0.172	0.007	0.18	1.08
13-23 <sub>1</sub>	Undil	0.5	0.866	1.120	0.254	0	0.25	0.50
13-23 <sub>2</sub>	3/10	1.0	0.690	0.900	0.210	0.018	0.23	0.77
13-23 <sub>3</sub>	'	1.0	0.606	0.818	0.212	0.020	0.23	0.77
13-23 <sub>4</sub>		1.0	0.760	0.964	0.204	0.018	0.22	0.73

Subnumerals indicate course numbers

precipitated an amount of antibody roughly equal to that found as agglutinin either with broth or agar grown suspensions. The polysaccharide from agar cultures, however, precipitated an amount of anticarbohydrate greatly in excess of the agglutinin nitrogen. Parallel experiments with agar itself showed that horse serum I 60 contained 0.38 mg per ml of protein nitrogen precipitable by agar and presumably formed in response to the antigenic stimulus of the traces of agar remaining on the agar grown bacilli used for the immunization (*cf* also Sordelli and Mayer (12)). The supernatant from the agar precipitate in serum I 60 was treated with influenza B polysaccharide giving an additional 0.60 mg per ml of anticarbohydrate. Thus, added to the amount precipitated by agar alone equaled 0.98 mg per ml the same quantity of nitrogen as was precipitated by the crude polysaccharide from the agar cultures. Influenza B polysaccharide prepared from such cultures therefore contains much soluble agar and this is also borne out by the relatively large amounts required for complete precipitation of the type specific antibody.



5 *Purification of Antibody in Type B Anti-Influenza Rabbit Sera*—In Table V are given data on the purification of rabbit antibody to *H influenzae*, type B, by the method recently published from this laboratory for antipneumococcus sera (13). The sera are allowed to stand in the cold, centrifuged, and diluted with several volumes of water. The total globulin is then precipitated by addition of an equal volume of sodium sulfate solution, saturated at 35–38°C. All water, materials, etc., are specially sterilized for intravenous injection and the precipitated globulin is collected sterily in a Sharples supercentrifuge, dissolved in water and saline, centrifuged from any remaining particles, and filtered through a Chamberland L2 filter. The solution is finally warmed to 60° for one-half hour as was recommended by Goodner, Horsfall, and Dubos for processed antipneumococcus rabbit sera (14). From the agglutinin content of the original serum pools and the resulting globulin solutions it is evident that substantially all of the antibody present is recovered in this method. Since any analytical error is multiplied several hundredfold in calculating the total antibody content in several hundred milli-

TABLE V

*Data on Antibody Globulin Prepared for Therapeutic Use from Anti-Haemophilus influenzae, Type B Rabbit Sera*

Preparation	Volume	Agglutinin N content	Precipitin N content	Recovered antibody globulin				Volume of recovered globulin	Agglutinin recovery	Precipitin recovery
				Total N	Agglutinin N	Precipitin N	Agglutinin N Total N × 100			
	of rabbit serum pool used									
	ml	mg	mg	mg per ml	mg per ml	mg per ml	per cent	ml	per cent	per cent
C	170	31	17		0.20	0.08		135	87	65
D	400	252		2.92	0.48	0.30	16	510	97	
E	380	137		3.38	0.48		14	305	107	

liters of serum, and, in addition, the total serum and antibody solution volumes were only approximately measured, it is clear that the figures might fluctuate rather widely around 100 per cent.

#### DISCUSSION

The data summarized in the tables illustrate the application of quantitative, absolute methods of agglutinin and precipitin analysis to horse and rabbit antisera to *H influenzae*, type B. Table I presents in outline an immunization procedure that has led to the production of sera of greatly improved antibody content. In Table II it is shown how total agglutinin may be determined with formalin-killed suspensions of the homologous strain in the mucoid phase and how group agglutinins may be estimated with heterologous or R strains. It is also shown in this table and in Table IV how, once the actual antibody content of the rabbit sera originally on hand was known, it was possible to increase the agglutinin and precipitin

content five to ten times by improved methods of immunization and frequent analytical control. The sera now being obtained with a content of 1 mg of antibody nitrogen per ml compare quite favorably with average rabbit antipneumococcus sera. In the anti-influenza sera, also, the principal antibody present is anticarbohydrate, just as in the type specific anti-pneumococcus sera. Whether or not the chief protective antibody is anticarbohydrate is less clear than in the case of the antipneumococcus sera, but it is hoped that studies now in progress will answer this question.

Of the various type B (V, Dawson (15)) suspensions used the formalin killed proved most satisfactory, and these absorbed approximately the same amount of antibody from the sera whether the bacilli originated from broth or agar cultures. Optimal conditions for capsule formation (6 hours' growth on Levinthal agar) favored the removal of anticarbohydrate from the sera. Microscopic examination showed that much of the capsular material was lost when young cultures were killed by heat, and such suspensions failed to remove agglutinins completely when added to sera in amounts comparable to those of the formalin killed suspensions which absorbed the sera completely in a single agglutination.

Table III summarizes the precipitin content of a single horse serum and of a number of rabbit sera. Very much less specific polysaccharide prepared from broth cultures was required to precipitate the antibody than was needed of samples prepared from agar cultures, so that it is evident that the latter preparations were grossly contaminated with soluble agar derivatives.<sup>4</sup> The total amount of antibody precipitable with the preparations from the two sources was almost the same in the case of the rabbit antisera, but in a horse serum (Table III) the antibody precipitated by the broth polysaccharide agreed quite well with the agglutinin value, while far more nitrogen was precipitated by the carbohydrate from the agar cultures. That the excess consisted of anti-agar, as noted in other instances by Sordelli and Mayer (12), was shown by the far larger amount of nitrogen precipitated from the serum by dilute agar solution than was thrown down by the same agar solution from the serum of another horse which had been injected solely with a protein. After absorption with agar the anti-influenza horse serum still showed an almost undiminished content of type B anticarbohydrate nitrogen. Possibly the slight excess of precipitin over agglutinin shown by the broth polysaccharide might be traced to antigenic broth components remaining in the preparation.

<sup>4</sup> The complicated procedures necessary for the elimination of such impurities are well illustrated in a recent paper by Miles and Pirie (16) on brucella antigens.

While the anti-influenza type B rabbit sera contained little anti-agar the proportion of anticarbohydrate precipitin to total antibody (agglutinin) appeared to vary rather widely. In general at least three-quarters of the antibody was accounted for as anticarbohydrate, and presumably the remainder consisted of antibodies to the somatic components of the bacillus.

Table IV shows the results of serial courses of immunization of two groups of rabbits (12 each, at the start). The details of the immunization procedure are given in section 1 of the experimental part and in Table I. Since most of the serum obtained was needed for therapeutic purposes the sera of individual animals were not analyzed. It appears, however, from the successive values obtained in the two groups, that the injection of massive amounts of type B organisms in the M phase offers little advantage over the use of more moderate amounts, as practised in the case of rabbits 1 to 12. However, the experiments must be repeated before a definite conclusion may be drawn.

From Table V it will be noted that substantially all the antibody in the rabbit sera may be recovered in the total globulin according to the simple procedure given in reference 13. Antibody prepared in this way has been repeatedly administered intravenously with no disturbing reactions. It would, however, doubtless be advisable to remove the sodium sulfate present by dialysis before intrathecal injections were attempted.

#### SUMMARY

1 The quantitative, absolute methods of agglutinin and precipitin analysis previously developed for antipneumococcus sera have been shown to be applicable to horse and rabbit anti-influenza type B sera and plasmas.

2 With the aid of these methods and improved immunization schedules the antibody content of the rabbit sera has been increased five to ten times.

3 The method recommended for the purification of rabbit antipneumococcus antibody has also been found applicable to rabbit anti-influenza type B sera.

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# THE SELECTIVE ACTION OF SULFANILAMIDE ON THE PARASITES OF EXPERIMENTAL MALARIA IN MONKEYS IN VIVO AND IN VITRO

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## PLATE 1

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Previous work has shown that the administration of as little as 1 gm. of sulfanilamide by mouth will completely eradicate both acute and chronic *Plasmodium knowlesi* infections in rhesus monkeys (1). The evidence of cure in these cases was (a) the permanent disappearance of parasites from the circulating blood, (b) the failure to reproduce the infection in normal monkeys by the subinoculation of fresh blood from treated animals, (c) the failure of treated monkeys to relapse following splenectomy, and (d) the successful reinfection of treated monkeys with the homologous parasite. These findings were of special interest because untreated *P. knowlesi* malaria in monkeys is an infection that is almost invariably fatal, and in this respect it represents one of the most virulent malaria infections in either human beings or experimental animals.

On the other hand, it has been the experience in this laboratory, as well as of most other workers, that sulfanilamide is an inferior therapeutic agent in human malaria (2-4). The drug has also failed to exert any demonstrable influence, even with excessive doses, in canaries and chicks infected with *Plasmodium cathemerium* and *Plasmodium lophurae*, respectively (5).

From the above mentioned findings it has been impossible to determine whether the activity of the drug varied from host to host, or whether it exerted its influence according to the species of plasmodium encountered. In order to elucidate this point, additional studies on the action of sulfanilamide on two plasmodial infections in a common host and on the effect of the drug on the metabolism of the parasites *in vitro* are here reported.

## Materials

The host used was the rhesus monkey and the two species of parasites employed were *Plasmodium knowlesi* and *Plasmodium mui* (6).

The minimal infective dose for *P. knowlesi* is probably between one and ten parasites (7), and since there are no known insect vectors, infected blood was used for the inoculations. Infections thus established, unless interrupted early with antimalaria drugs, usually terminate in the death of the animal. At the time of death 50 per cent or more of the red blood cells contain parasites, and there is a profound anemia.

*P. mui* produces a moderately severe infection following the injection of infected blood. The parasite density at the height of the acute disease rarely exceeds 15 per cent of the normal red blood cells, and the acute attack usually subsides spontaneously into a chronic infection of indefinite duration.

Mixed infections in monkeys to be used for treatment were produced by injecting viable *P. knowlesi* parasites into monkeys with chronic *P. mui* infections. Mixed infections in the controls were obtained in three ways: (a) the simultaneous injection of the two parasites, (b) superinfection of monkeys harboring *P. mui* infections with *P. knowlesi* parasites, and (c) superinfection of monkeys with chronic *P. knowlesi* infections with *P. mui* parasites.

For treatment of the infected monkey sulfanilamide (para-amido-benzene sulfonamide) was used, and the drug was always given by mouth in 1 gm doses.

### *Effect of Sulfanilamide on Simple P. knowlesi and P. mui Infections*

The dramatic manner in which sulfanilamide effects a cure of *P. knowlesi* infections in *rhesus* monkeys was briefly summarized above.

*Experiment 1*—In order to test its effect against *P. mui* infections, monkeys 1, 2, and 3 were injected and treated as shown in Table I. Normal monkeys 1 and 2 were each inoculated intra-abdominally with 1 cc of parasitized fresh blood. Daily blood smear examinations revealed typical parasites in both monkeys by the 6th day. Monkey 1 was then given three daily 1 gm doses of sulfanilamide by mouth, while monkey 2 was kept as an untreated control. During the first 48 hours of the period of treatment of monkey 1 there was a slow increase in the parasite count. This was followed by a gradual decline to a point where plasmodia were absent from the smears. This picture persisted for approximately 2 weeks, when the parasites reappeared and steadily increased in number. Monkey 2, the untreated control, had a typical *P. mui* infection with the peak of the acute attack occurring on the 14th day. 6 weeks later, with both animals having identical parasite counts, monkeys 1 and 2 were each given three daily 1 gm doses of sulfanilamide, this being the second course of treatment for monkey 1 and the first for monkey 2. Again there was a temporary reduction in circulating parasites in both monkeys, and smears were never negative for more than a few days at a time. Monkey 3, used to repeat the experiment, had a chronic *P. mui* infection of 3 months' duration. Its parasite count was 15<sup>1</sup> at the time when sulfanilamide was first given. Three daily 1 gm doses produced no significant effect on the parasite density until 48 hours after the third dose, when there was a diminution. For 3 weeks the daily smears showed only an occasional parasite from time to time, and the count then returned to its pretreatment level.

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<sup>1</sup> Parasite counts given in this paper refer to the number of parasitized cells per 10,000 red cells.

The outcome of these results is seen in Table I. It was observed that sulfanilamide exerted only a temporary influence on *P. mui* in rhesus monkeys, regardless of whether they were treated during the acute, sub-acute, or chronic stages of the disease. This was true even with dosage in excess of the amount found necessary to eradicate the more virulent *P. knowlesi* infections in the same species of monkey.

### *Effect of Sulfanilamide in Mixed Infections*

To obviate the influence of such factors as individual host variation, differences in the composition of the drug used on different days, and variations in the number and stage of parasites in the inoculum, an attempt was made to demonstrate the selective activity of sulfanilamide in mixed infections in the same experimental animal. One should thus have a better opportunity to observe the effects directly or indirectly on the parasite itself.

*Experiment 2*—Monkeys 4 and 5, shown in Table I, were suffering with chronic *P. mui* infection and each was superinfected with 10 000 *P. knowlesi* parasites. When the total parasite count of monkey 4 had increased from 16 to 160, and both species of parasite were identified in the smears from morphological characteristics, the animal was given a 1 gm dose of sulfanilamide by mouth. On the 3rd day following treatment parasites completely disappeared from the circulating blood. During this interval there was marked phagocytosis of malaria parasites by the circulating macrophages, as shown in Figs 1 A and 1 B, some of which contained as many as ten parasitized red cells. Parasites did not reappear until 27 days after treatment when they were identified as *P. mui* morphologically. This fact was confirmed by subinoculation into a normal monkey, which developed a typical chronic *P. mui* infection from monkey 4. Monkey 5 remained untreated until the parasite count was 2,880 and the red count was 2,100 000. The infection was predominantly *P. knowlesi* although there were also present some of the *P. mui* variety. At this point monkey 5 was so critically ill that it was transfused with 50 cc of citrated normal monkey blood before being given 1 gm of sulfanilamide by mouth. On the following day about half the cells were parasitized (count 4,530), and a second transfusion of 40 cc of normal blood was given along with 2 gm of the drug. The parasite count dropped to 46 within 24 hours and was zero in 48 hours. During this period there was great activity of circulating macrophages in the phagocytosis of infected cells as shown in Fig 1 A and 1 B. Parasites outside the macrophages appeared distorted and were poorly stained. Thereafter there was an absence of parasites for 34 days and when they reappeared they were identified morphologically as *P. mui*. This identification was confirmed as with monkey 4, by subinoculation of blood into a normal monkey, and a resultant simple *P. mui* infection.

The results seen in Table I showed that sulfanilamide, either directly or indirectly, had the ability to remove a virulent plasmodial infection and was practically without influence on the milder *P. mui* infection in the same experimental animal.



TABLE I

*Results of Sulfanilamide Therapy on Simple P mui and Mixed P knowlesi and P mui Malaria Infections in Rhesus Monkeys and Untreated Controls*

Monkey No	History of previous infection		Parasite used for inoculation	Treatment with sulfanilamide		Results
	Kind	Duration		Time after last inoculation at beginning of treatment	Parasite count at beginning of treatment	
Treated <i>P mui</i> Infections						
1	Normal monkey	— <i>days</i>	<i>P mui</i>	5 49*	+ +	Temporary suppression of infection
2	Normal monkey	—	<i>P mui</i>	49	12	Temporary suppression of infection
3	<i>P mui</i>	90	—	64	32	Temporary suppression of infection
Treated Mixed Infections						
4	<i>P mui</i>	110	<i>P knowlesi</i>	14	160	Disappearance of <i>P knowlesi</i> and temporary suppression of <i>P mui</i>
5	<i>P mui</i>	115	<i>P knowlesi</i>	10	2,880	Disappearance of <i>P knowlesi</i> and temporary suppression of <i>P mui</i>
Untreated Mixed Infections (Controls)						
6	<i>P mui</i>	68	<i>P knowlesi</i>	—	—	Died 11th day of <i>P knowlesi</i> infection
7	<i>P mui</i>	75	<i>P knowlesi</i>	—	—	Died 7th day of <i>P knowlesi</i> infection
8	<i>P knowlesi</i>	100	<i>P mui</i>	—	—	Typical <i>P mui</i> infection
9	<i>P knowlesi</i>	115	<i>P mui</i>	—	—	Typical <i>P mui</i> infection
10	Normal monkey	—	<i>P knowlesi</i> and <i>P mui</i>	—	—	Died 13th day of <i>P knowlesi</i> infection
11	Normal monkey	—	<i>P knowlesi</i> and <i>P mui</i>	—	—	Died 11th day of <i>P knowlesi</i> infection

\* Same monkey treated twice with sulfanilamide

+ indicates less than one parasite per 10,000 R B C

### *Effect of Mixed Plasmodial Infection in Untreated Monkeys*

In order to demonstrate that the drug was responsible for the disappearance of the more virulent parasite and not an antagonistic effect in mixed infections, monkeys were infected with both types of parasites and their infections allowed to go untreated

*Experiment 3*—The details and results of this experiment are summarized in Table I. Monkeys 6 and 7 with chronic *P. mui* infections of 68 and 75 days' duration were inoculated with 10,000 *P. knowlesi* parasites. Both monkeys showed the presence of a mixed infection on the 5th day after inoculation and died on the 11th and 7th days, respectively. Monkeys 8 and 9 with chronic *P. knowlesi* infections established by quinine some months previously, were similarly infected with viable *P. mui* parasites. In both instances the inoculated monkeys experienced a typical mild *P. mui* infection. Normal monkeys 10 and 11 were each inoculated with a mixture of 10,000,000 *P. mui* and 10,000 *P. knowlesi* parasites. Both monkeys had mixed infections on the 7th day and died on the 13th and 11th day, respectively, the *P. knowlesi* parasites having multiplied at their usual rate overshadowing the mild infections.

The results of these controls, summarized in Table I, show that in untreated mixed infections there is no evidence of an antagonistic effect between the two plasmodia.

#### *The Effect of Sulfanilamide on the Metabolism of the Parasites in Vitro*

The difference in the reaction of plasmodial infections to sulfanilamide in a common host was taken as evidence that the drug acted directly on the parasite independently of the host. An attempt was made to find whether the same difference would make itself manifest when sulfanilamide was added to the parasites *in vitro*. This was done by testing the effect of sulfanilamide on the respiration of the two parasites in a Warburg manometer.

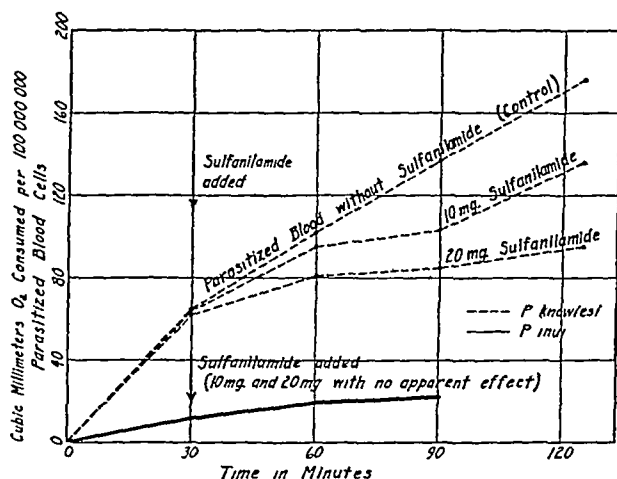
The parasites were obtained by bleeding monkeys with well advanced infections. In order to obtain *P. mui* infections that were comparable in intensity to *P. knowlesi*, it was necessary to use splenectomized monkeys. Heparinized infected blood was centrifuged and the brown parasite substance, which has a lower density, was removed from the top layer of the cellular material. This was then resuspended in the same monkey's serum in the ratio of one part of parasites to three parts of serum. The number of reticulocytes and leukocytes in the mixture was below the concentration at which their metabolism could interfere with the results. The system used was essentially that of Christophers and Fulton (8), with the exception that glucose was added to the medium. The sulfanilamide was put in a side arm with Ringer solution at the end of a 30 minute control run. To absorb the CO<sub>2</sub> produced, 0.3 cc. of 40 per cent KOH was put in the center cup. Glycolysis was run in the same medium with NaHCO<sub>3</sub> added.

The results as shown in Text fig. 1 are based on 100 million parasites, and it was found that the oxygen consumption for *P. knowlesi* was ap

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The determinations on the metabolic rate of the parasites were done by Mr. Charles Kensler through the courtesy and in the laboratories of Dr. C. P. Rhoads at The Rockefeller Institute for Medical Research.

proximately six times greater than for the same number of *P. vivax* parasites. Although the concentration of sulfanilamide (20 mg. in system) was approximately one-third that used to cure a monkey, there was a marked paralyzing effect on the respiration of the parasite. With 10 mg. of sulfanilamide the effect was definite, but to a less degree. There was no observable effect on the *P. vivax* parasites by the same concentrations of the drug. The anaerobic  $\text{CO}_2$  production of both parasites was unaffected by sulfanilamide. The R.Q. of *P. knowlesi*, confirming Christophers and Fulton (8), and of *P. vivax* was found to be slightly less than one.



TEXT-FIG 1 Influence of sulfanilamide on the rate of oxygen consumption of *P. knowlesi* and *P. vivax* parasitized red cells *in vitro*

### Effect of Sulfanilamide against *P. knowlesi* in Other Hosts

The next point taken up was whether sulfanilamide is effective against the parasites regardless of the host. Human volunteers with general paresis and resistant to therapeutic *vivax* malaria were inoculated with *P. knowlesi* infected blood and subsequently treated with the drug. Although the infections were mild, they disappeared promptly after the administration of sulfanilamide, but the extremely variable character of untreated *P. knowlesi* infections in man does not allow for more than inferential evidence of the action of the drug.

Two *cynomolgus* monkeys were inoculated with blood infected with *P. knowlesi*, and in this instance also the resultant infection in untreated controls was so mild and transitory that no definite conclusions can be drawn.

## EXPLANATION OF PLATE 1

FIG 1 A High percentage of parasitized red blood cells with untreated, mixed *P knowlesi* and *P. mui* infections

FIG 1 B Macrophages with phagocytosed infected red cells in blood from same monkey 48 hours after sulfanilamide therapy

## EXPLANATION OF PLATE 1

FIG 1 A High percentage of parasitized red blood cells with untreated, mixed *P knowlesi* and *P. vivax* infections

FIG 1 B Macrophages with phagocytosed infected red cells in blood from same monkey 48 hours after sulfanilamide therapy

## EXPLANATION OF PLATE 1

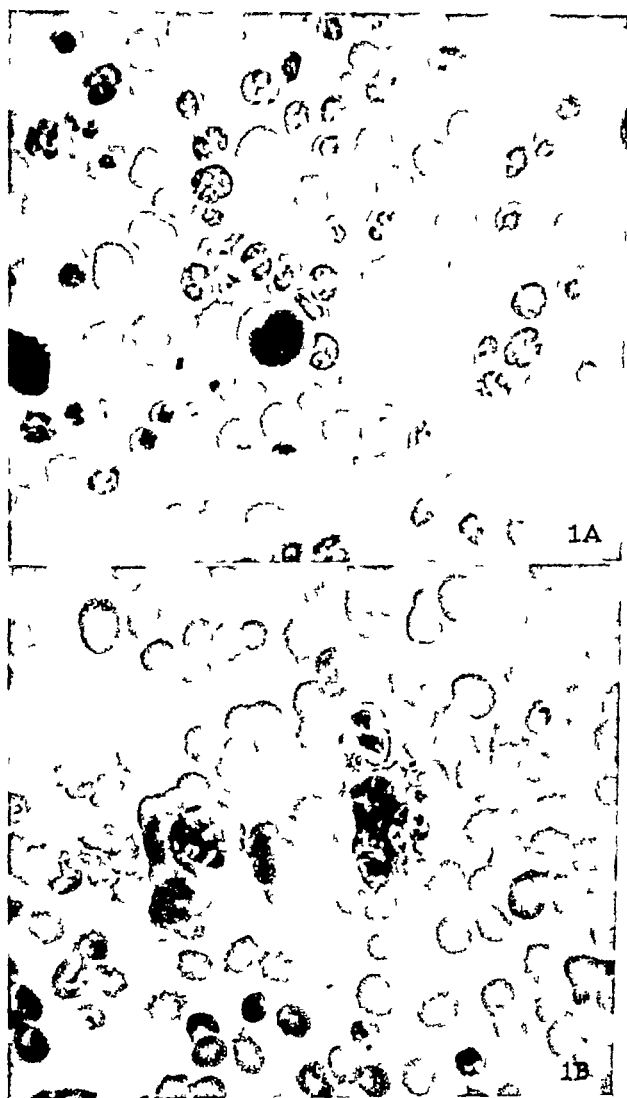
FIG 1 A High percentage of parasitized red blood cells with untreated, mixed *P knowlesi* and *P. mui* infections

FIG 1 B Macrophages with phagocytosed infected red cells in blood from same monkey 48 hours after sulfanilamide therapy

## EXPLANATION OF PLATE 1

FIG 1 A High percentage of parasitized red blood cells with untreated, mixed *P knowlesi* and *P vivax* infections

FIG 1 B Macrophages with phagocytosed infected red cells in blood from same monkey 48 hours after sulfanilamide therapy



Photographed by Joseph B. Haulenbeek

(Coggeshall Selective action of sulfanilamide in malaria)





# PERMEABILITY OF THE HUMAN PLACENTA TO ANTIBODIES\*†

## A QUANTITATIVE STUDY

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A problem of some importance and one which soon attracted the attention of immunologists is the source of serum antibodies during the neonatal period. In early experiments on various animal species, some investigators (1-3) noted a passage of antibodies from mother to newborn through the placenta, while others (4, 5) reported the placenta to be impermeable to antibodies. In cattle it was found that the antibodies of recently born calves are derived mainly from the colostrum (5). For example, Smith and Little (6) showed that as many as 75 to 80 per cent of calves that failed to obtain colostrum by nursing succumbed to a generalized *Bacillus coli* septicemia.

To explain these contradictory findings, Kuttner and Ratner (7) suggested that there was a correlation between the anatomical structure of the placenta and its permeability to antibodies. According to their idea, the more dense the barrier between the circulations of mother and fetus the less transmission of antibodies occurs. In instances where there is no significant placental transfer of antibodies, as in cattle, the colostrum plays the major rôle. In general, the importance of the colostrum is inversely proportional to that of the placenta under the theory of Kuttner and Ratner. For a more complete discussion of the passage of antibodies and heterologous proteins through the placenta, colostrum and milk, see Ratner *et al* (8).

In man, since the chorionic villi bathe in the maternal blood, it would be expected under the theory just enunciated that the placental transfer of antibodies would be the major source of antibodies for the newborn. At

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† With the technical assistance of Miss Eve Sonn.

any rate, the common observation that infants artificially fed from birth thrive as well as breast-fed infants proves that the colostrum is not essential for their well being. In this connection may be cited the observations of Kuttner and Ratner (7) on the antitoxin content of colostrum and cord blood.

For the investigation of the placental transfer of immune bodies in human beings various antibodies have been used. In 1895, only a few years after the announcement of the discovery of diphtheria antitoxin by von Behring and his associates, Fischl and von Wundschheim (9) demonstrated the presence of diphtheria antitoxin in a large proportion of cord bloods. Shortly thereafter, Schumacher (10) presented evidence for the passage through the human placenta of agglutinins for typhoid bacilli. Halban and Landsteiner (11) made a comparative study of heterohemolysins, heterohemagglutinins and antitoxins in maternal and umbilical cord sera and found them always to exist in considerably smaller quantities in the cord sera. Evidence has been adduced more recently for the passage of isoagglutinins through the placenta (12, 13). An unexplained observation, however, was that frequently isoagglutinins were lacking from cord blood even when the infant belonged to the same group as the mother. It occurred to the present authors that the reason for this behavior might be found by making quantitative studies on the isoagglutinins in maternal and cord bloods. Moreover, by comparing the titers of a variety of antibodies it might be possible to establish an "index of permeability" for the human placenta, or what might perhaps more properly be called a "coefficient of distribution" of antibodies between mother and fetus.

### *Methods and Materials*

For the present study the following antibodies were selected: the isoagglutinins  $\alpha$  and  $\beta$ , heteroagglutinins for rabbit and sheep blood and syphilitic reagin. The titrations for the hemagglutinins were carried out in the usual way in small test tubes by mixing progressively doubled dilutions of the serum being examined with equal volumes of the dilute test blood suspensions (14). The titrations of syphilitic reagin were made in a similar way except that the tests were set up on paraffin-ringed slides (15). The test antigens used for titrating the syphilitic reagin were the so called Kline diagnostic and exclusion antigens for heated serums. Sample protocols are given in Table I. For the sake of uniformity, in this table the strengths of the agglutination reactions both for red cells and Kline antigen have been rated up to maximum of four plus, depending on the size of the clumps. The titers were taken equal to the reciprocal of the highest dilution of serum still giving a reaction visible to the naked eye (two plus or more).

### RESULTS

In Table I it will be seen that the ratios (designated for convenience by the letter R) of the titers of the various antibodies in the maternal blood

TABLE I  
Titration of Antibodies in Maternal Blood and Umbilical Cord Blood

Case No	Test antigens	Dilutions of maternal serum								Dilutions of cord serum						Ratio (R) of titers	
		1 1	1 2	1 4	1 8	1 16	1 32	1 64	1 128	Titer	1 1	1 2	1 4	1 8	1 16		Titer
1	B cells	+++	+++	+++	++	-	-	-	-	8	++	-	-	-	-	1	8
	Rabbit cells	+++	+++	+++	+++	+++	++	-	-	32	+++	++	-	-	2	16	
	Sheep cells	+++	+++	+++	-	-	-	-	-	1	-	-	-	-	0	-	
	D*	+++	+++	+++	-	-	-	-	-	4	-	-	-	-	0	-	
	E	+++	+++	+++	++	-	-	-	-	8	+++	-	-	-	1	8	
2	A <sub>1</sub> cells	+++	+++	+++	+++	+++	++	-	-	32	+++	-	-	-	1	32	
	B cells	+++	+++	+++	+++	++	-	-	-	16	+++	++	-	-	2	8	
	Rabbit cells	+++	+++	+++	+++	+++	++	-	-	32	+++	+++	++	-	4	8	
	Sheep cells	+++	+	-	-	-	-	-	-	1	-	-	-	-	0	-	
	group O																
3	A <sub>1</sub> cells	+++	+++	+++	+++	+++	++	++	-	64	+++	+++	+++	++	-	8	8
	B cells	+++	+++	+++	+++	+++	+++	++	-	64	+++	+++	+++	+++	-	8	8
	Rabbit cells	++	+++	+++	+++	+++	+++	++	-	64	+++	+++	+++	+++	-	8	8
	Child group O																
	group O																

\* D = Kline diagnostic antigen E = Kline exclusion antigen

to the titers of the corresponding antibodies in the fetal blood vary between 8 and 32 in the nine instances where a value for R could be obtained. The experiments completed to date have yielded a total of 56 ratios including the nine listed in Table I, and in this entire series the value for R has never fallen below 4 nor exceeded 32. One thing of interest which became evident early in the study was that the variation of the value of R from one individual to another was not significantly greater than the variation of R for different antibodies in the same individual.

It is important to bear in mind that the observed or apparent variation in the value of R must be considerably greater than the actual one on account of the limitations of the technique. Since the dilutions of serum are progressively doubled, it is clear that the error must be close to 50 per cent, assuming that the results are as accurate as they possibly can be. As a matter of fact the error is often greater, since it is not unusual to find on repetition of titrations a difference of one tube.

It is possible by making certain assumptions to calculate the actual variations in the values of R from those observed. Let us assume for example, that R has the constant value 12, and the error of the titration technique used is  $\frac{1}{2}$  of a serial dilution. If the true titer of a particular antibody in the mother be set equal to  $12\tau$ , then the titer of the corresponding antibody in the cord blood would be  $\tau$ . In the tests, the titer of the maternal antibody might be found to lie anywhere between  $9\tau$  and  $18\tau$ , whereas the observed antibody titer for cord blood would fall between  $\frac{3}{4}\tau$  and  $\frac{3}{2}\tau$ . The observed value of R instead of being 12 could therefore have any value between  $9\tau - \frac{3}{2}\tau$  and  $18\tau - \frac{3}{4}\tau$ , that is, between 6 and 24. Conversely, if R is found to vary between 6 and 24 and it is known that the method has an error of  $\frac{1}{2}$  dilution, one would have to conclude that R actually does not vary at all, its true value being probably equal to 12.

In the present study, as already mentioned, the calculated R's ranged from 4 to 32, which closely approximates the range in the hypothetical case just described. It is not unreasonable to conclude, therefore, that in human beings antibodies, at any rate those investigated here, are distributed at the time of birth so that the ratios of their titers in maternal and fetal bloods are relatively constant. The true value of R lies somewhere between 8 and 16. As additional evidence in support of these conclusions can be mentioned some of our experiments which revealed that the observed variations in the value of R in different individuals, or between different antibodies in the same individual, were no greater than the variable results obtained for the value of R for a particular antibody in a particular mother and child on repeating the titrations several times.

It now becomes clear why in certain cases in which the mother and child belong to the same group isoagglutinins are not demonstrable in the cord

blood If the titer of the isoagglutinin in the mother's blood is much less than 8, there will not be enough isoagglutinin present in the cord blood to be detectable with the standard technique used However, that does not indicate the failure of placental transfer of the isoagglutinin For example, in one of our cases where the mother belonged to group A and child to group O, the titrations set up in the usual way (readings after 2 hours at room temperature) showed the titer of the  $\beta$  agglutinins in the maternal blood to be 4 and  $\beta$  agglutinins were apparently absent from the cord blood, but when the readings were taken after standing in the ice box overnight the respective titers were 8 and 1 The claims made by some writers that the natural heteroagglutinins for sheep cells do not pass through the placenta can easily be answered in a similar way, since as indicated in Table I the titer of the agglutinins in the mother is usually considerably below 8<sup>1</sup>

#### COMMENT

It is of interest to contrast the results obtained in a previous study by Wiener and Derby (17) with those given above In attempting to ascertain whether syphilitic reagin in the spinal fluid of patients with neurosyphilis is formed locally or derived from the blood by filtration, parallel titrations were made of isoagglutinins and syphilitic reagin in the blood and spinal fluid of such patients It was found that even when the titer of reagin in the spinal fluid equalled that of the blood and the titer of the isoagglutinin in the blood was much higher than that of the reagin, no isoagglutinins could be demonstrated in the spinal fluid This indicated that the reagin in spinal fluid is at least in large part formed locally On the other hand, the fact that the ratio of the reagin titers of maternal to cord blood is equal to the value of the ratio, R, for other antibodies such as the isoagglutinins, indicates that syphilitic reagin in umbilical cord blood, like other antibodies, is derived from the maternal blood by filtration through the placenta

A question of special interest which is related to the present study is whether or not sensitizing bodies (the so called reagins of Coca) pass through the placenta in a similar fashion According to Coca (18), reagins differ

<sup>1</sup> In the present study the behavior of bacterial agglutinins with regard to the placenta was not studied, but it is reasonable to assume that it would be similar to that of hemagglutinins No direct evidence in support of this assumption is available but it is of interest to cite a remark found in a report by Toomey (16) on agglutinins for *B coli* in maternal and cord serum This author remarks that the maximum titer of these bacterial agglutinins in the maternal blood was 640 whereas for cord blood the maximum was 80 The value for R for agglutinins for *B coli* calculated from these maximum values would be 8, which agrees with that obtained for the hemagglutinins

at timed intervals between 0 and 50 per cent oxidation. The tube is kept in a 30° bath and is mixed and wiped clean before each reading. A final reading is taken after oxidation is complete, *i e*, after an interval 3 or 4 times that required for 50 per cent oxidation. If available, 5  $\mu$ l of an active xanthine oxidase preparation are added to complete the reaction in a few minutes. Care is taken that the working standard (quinine sulfate in 0.1 N H<sub>2</sub>SO<sub>4</sub>) does not change temperature over the experimental period.

If the change in fluorescence is not linear with time, the initial velocity is calculated graphically. (The rate may fall off, due to product inhibition (4).) 1 ml of substrate is  $5 \times 10^{-6}$  mmole. Thus, if 1 mg of liver caused a change in fluorescence of 1 galvanometer division per minute and the change for 100 per cent oxidation was 80 divisions, the tissue enzyme activity would be  $1/80 \times 5 \times 10^{-6} \times 60 \times 10^6 = 3.75$  mmoles per kilo per hour.

The Michaelis constant for xanthine oxidase with aminohydroxypteridine is quite small, so that the enzyme is saturated throughout most of the course of the reaction (4). Therefore, the time required to oxidize a given fraction of the substrate will vary with the substrate concentration. The substrate level chosen is low enough to permit a good percentage rate without requiring an amount of tissue that would produce marked turbidity and consequent quenching. It is also low enough to prevent self-quenching. On the other hand, the substrate level is high enough to provide strong fluorescence and avoid troublesome optical blanks. The procedure used and the method of calculation whereby each sample is its own standard automatically correct for possible quenching or contribution of fluorescence from the enzyme sample.

In comparison with methods based upon oxygen consumption, this procedure does not suffer from difficulties arising from large endogenous blanks (5), and, in comparison with methods based on uric acid formation or disappearance of xanthine (6, 7), it is not complicated by tissue uricase. Technically it is simple and rapid. Because it is a fluorometric method, it is more sensitive than other current procedures, in spite of the fact that the absolute rate of oxidation with the pterin substrate is only about a fifth of the rate with xanthine (4).

*Amino Acid and Glycolic Acid Oxidases*—The oxidases for D-amino acids, L-amino acids, glycine, and glycolic acid were all measured by similar spectrophotometric procedures. The keto acids formed from D-alanine, L-leucine, glycine, or glycolic acid were condensed with quinoxalylhydrazine and measured in the Beckman spectrophotometer at 305 m $\mu$  (8, 9).

The activities fall during prolonged incubation, particularly in riboflavin-deficient tissues. This is probably due in part to dissociation of the

prosthetic groups from the enzymes. Analytical conditions were therefore established which permit short incubation periods to minimize this effect. It is believed that the result is a much closer approximation to the activity in the original tissue than was possible with earlier methods. The details will be published separately.

### Results

*Normal Levels of Flavin Enzymes and Coenzymes*—The FAD and FMN normal values are slightly lower, but in general agree with earlier data (1). The very low levels of both coenzymes in brain are striking (Table I), and the high level of FMN in kidney and the low level in heart are particularly noteworthy. None of the enzymes measured thus far in kidney account for a function of the high level of renal FMN.

Flavin enzyme levels differ greatly from tissue to tissue (Tables I to III). Liver and kidney have the highest levels of the enzymes measured and brain the lowest, while heart is intermediate. D-Amino acid oxidase is highest in kidney, with liver about one-tenth and both heart and brain about 100th as high. L-Amino acid oxidase is 3 times as high in kidney as in liver. Xanthine oxidase is too low to measure in rat brain, but in heart there is one-tenth as much as in liver. Glycolic acid oxidase is relatively high in liver (Table III), 100 times lower in kidney (not shown), and not measurable in other tissues. In contrast, glycine oxidase is twice as high in kidney as in liver.

The absolute activity of DPNH dehydrogenase as measured with ferricyanide is greater than that of any other flavin enzyme studied (Table II). Liver contains twice as much DPNH dehydrogenase as either kidney or heart, and 10 times as much as brain. The low value in brain is particularly surprising since the oxygen consumption of brain (0.08 mole or 0.32 electron equivalent per kilo, wet weight, per hour (10)) is comparable to that of liver (0.07 mole or 0.28 electron equivalent per kilo, wet weight, per hour (11)). This might be interpreted as indicative of some difference in electron transport in brain and liver. The DPNH dehydrogenase activity as measured in brain with ferricyanide is equivalent to about 3 times the oxygen consumption, whereas in liver it is equivalent to 25 times the oxygen consumption. Therefore, the DPNH dehydrogenase has enough capacity to handle the electron transport in brain, although there is much less margin of safety than in other tissues. It is perhaps not surprising that DPNH dehydrogenase activities measured with different electron acceptors are not strictly proportional from tissue to tissue. With cytochrome *c* as acceptor, liver is 3.3-fold as active as kidney, whereas with ferricyanide as acceptor it is only 1.8 times as active. Differences in the activity of intermediate components between cytochrome *c* and the de-



TABLE I

*Levels of Riboflavin Enzymes and Coenzymes in Rat Tissues*

Each value is the average obtained on tissues from four rats in each group  $\pm$  the standard error of the mean. Calculations are based on wet weight. FMN and FAD are expressed as riboflavin in this and in Tables II to V.

Group*	FMN $\gamma$ per gm	FAD $\gamma$ per gm	DPNH dehydrogenase with		D-Amino acid oxidase  mmoles per kg per hr	Xanthine oxidase  mmoles per kg per hr	Protein  gm per kg
			Cytochrome c  mmoles per kg per hr	Dye†  mmoles per kg per hr			
Brain							
High riboflavin	0.68 $\pm$ 0.4	2.4 $\pm$ 0.13	0.28 $\pm$ 0.02	0.19 $\pm$ 0.01	3.1 $\pm$ 0.2		96 $\pm$ 2.7
Low riboflavin	0.56 $\pm$ 0.02	2.3 $\pm$ 0.04	0.29 $\pm$ 0.01	0.18 $\pm$ 0.01	2.3 $\pm$ 0.3		100 $\pm$ 1.7
Deficient	0.54 $\pm$ 0.01	2.1 $\pm$ 0.05	0.29 $\pm$ 0.03	0.17 $\pm$ 0.01	2.8 $\pm$ 0.1		102 $\pm$ 1.5
Weight control	0.71 $\pm$ 0.06	2.6 $\pm$ 0.08	0.29 $\pm$ 0.02	0.18 $\pm$ 0.01	2.6 $\pm$ 0.2		101 $\pm$ 2
Liver							
High riboflavin	5.7 $\pm$ 1.0	27.0 $\pm$ 1.2	4.0 $\pm$ 1.6	1.9 $\pm$ 0.7	26.4 $\pm$ 4.1	5.5 $\pm$ 0.5	182 $\pm$ 4.7
Low riboflavin	0.68 $\pm$ 0.1	11.8 $\pm$ 0.5	4.3 $\pm$ 1.1	2.2 $\pm$ 0.4	8.2 $\pm$ 4	4.5 $\pm$ 0.3	174 $\pm$ 3.7
Deficient	0.48 $\pm$ 0.1	11.8 $\pm$ 0.2	4.5 $\pm$ 1.1	2.6 $\pm$ 0.6	6.5 $\pm$ 1.4	3.1 $\pm$ 0.3	187 $\pm$ 8.9
Weight control	3.4 $\pm$ 0.3	30.2 $\pm$ 0.7	4.1 $\pm$ 1.3	2.2 $\pm$ 0.8	19.3 $\pm$ 5	4.0 $\pm$ 0.4	185 $\pm$ 5.6
Kidney							
High riboflavin	16.0 $\pm$ 0.3	19.1 $\pm$ 0.9	1.8 $\pm$ 0.29	1.1 $\pm$ 0.2	20.70 $\pm$ 5.8	2.0 $\pm$ 0.1	174 $\pm$ 9.0
Low riboflavin	10.2 $\pm$ 0.7	17.4 $\pm$ 0.7	1.6 $\pm$ 0.32	1.1 $\pm$ 0.4	19.73 $\pm$ 3.3	2.0 $\pm$ 0.2	170 $\pm$ 2.8
Deficient	6.7 $\pm$ 0.5	16.5 $\pm$ 0.5	1.7 $\pm$ 0.28	1.0 $\pm$ 0.1	14.61 $\pm$ 7.2	1.3 $\pm$ 0.1	159 $\pm$ 3.1
Weight control	11.4 $\pm$ 0.5	20.4 $\pm$ 1.0	1.9 $\pm$ 0.37	0.9 $\pm$ 0.2	17.51 $\pm$ 8.6	1.6 $\pm$ 0.2	161 $\pm$ 3.4
Heart							
High riboflavin	1.6 $\pm$ 0.2	19.7 $\pm$ 1.3	1.3 $\pm$ 0.16	1.0 $\pm$ 0.4	2.7 $\pm$ 0.8	0.5 $\pm$ 0.02	144 $\pm$ 7.2
Low riboflavin	1.2 $\pm$ 0.1	13.8 $\pm$ 1.1	1.0 $\pm$ 0.06	0.8 $\pm$ 0.2	1.5 $\pm$ 0.2	0.6 $\pm$ 0.03	139 $\pm$ 9.0
Deficient	1.2 $\pm$ 0.1	11.7 $\pm$ 0.2	1.0 $\pm$ 0.04	0.8 $\pm$ 0.3	1.7 $\pm$ 0.3	0.6 $\pm$ 0.04	142 $\pm$ 9.0
Weight control	1.2 $\pm$ 0	17.2 $\pm$ 0.7	1.3 $\pm$ 0.26	0.9 $\pm$ 0.1	1.6 $\pm$ 0.5	0.6 $\pm$ 0.04	129 $\pm$ 2.6

\* The average initial weight of each group was 60 gm and the average weight gain per rat per day was 1.7 gm for the deficient, 1.8 for the weight control, 4.3 for the low riboflavin, and 6.0 for the high riboflavin groups. Food intakes of 4.1, 3.4, and 2.1 gm produced a weight gain of 1 gm in the deficient, weight control, low, and high riboflavin groups, respectively.

† The dye was 2,6-dichlorobenzeneendo-3'-chlorophenol.

hydrogenase might easily explain this discrepancy, since ferricyanide taps into the electron transport chain below the point at which antimycin A is inhibitory (12)

*Effects of Deficiency, FMN, and FAD*—The findings confirm and extend

TABLE II  
*Electron Transport Enzymes in Tissues of Rats Depleted of  
Riboflavin for 12 to 13 Weeks*

Each value is the average of several analyses from the tissues of four or five rats  
Enzyme activities are expressed as moles per kilo of wet weight per hour

Tissue	FMN	FAD	DPNH dehydrogenase measured with		
			Cytochrome c	Dye*	Ferricyanide
	<i>γ per gm</i>	<i>γ per gm</i>			
Brain					
High riboflavin	0 79	2 27	0 31	0 19	0 97
S e m	0 05	0 11	0 04	0 02	0 07
Deficient	0 42	1 95	0 29	0 20	0 81
S e m	0 13	0 06	0 01	0 02	0 03
Liver					
High riboflavin	4 00	26 52	4 63	2 06	7 43
S e m	0 53	0 60	0 33	0 37	0 44
Deficient	0 19	8 03	3 89	2 21	6 63
S e m	0 08	0 25	0 16	0 22	0 29
Kidney					
High riboflavin	15 25†	20 65	1 56	0 92	3 28
S e m	0 69	0 69	0 18	0 11	0 30
Deficient	2 76	13 74	1 51	0 66	2 85
S e m	0 48	0 58	0 13	0 11	0 10
Heart					
High riboflavin	1 68	21 45	1 40	0 83	4 16
S e m	0 31	0 78	0 14	0 13	0 14
Deficient	1 01	7 41	0 92	0 64	3 56
S e m	0 12	0 31	0 10	0 09	0 17

S e m = the standard error of the mean

\* The dye was 2,6-dichlorobenzeneindo-3'-chlorophenol

† These are the results on three rats

an earlier report (1) Tissues differ a great deal in the rate and extent of fall in FAD and FMN levels FMN, in general, falls more rapidly than FAD (Table I, Fig 1) By 12 weeks (Table II) hepatic FMN has almost disappeared Curiously, cardiac FMN, which was initially low, falls only by a third even in severe deficiency Liver suffers the greatest loss in FAD (70 per cent), whereas brain FAD is diminished by only 15 per cent The inclusion of sufficient riboflavin in the diet to give 70 per cent maximal

TABLE III

*Regeneration of Flavin Enzymes in Depleted Rats after Administration of Riboflavin*

Each value is the average of duplicate analyses on the tissues of five or six rats  
Enzyme activities are expressed as millimoles per kilo per hour

Group*	Weight of organ	FAD	FMN + free ribo flavin†	Enzyme activity				
				Glycolic acid oxidase	Glycine oxidase‡	D-Amino acid oxidase	L-Amino acid oxidase	Xan thine oxidase
Liver								
	gm	γ per gm	γ per gm					
Deficient control	3.8	10.0	0.4	17	1.7	34	4.8	2.2
S e m	0.2	0.5	0.1	2	0.3	18	0.4	0.4
After dose, 0.5 hr	3.3	15.1	4.8	39	2.0	110	6.4	3.3
S e m	0.3	0.9	0.8	6	0.6	22	0.8	0.5
2 hrs	3.7	18.9	1.5	70	3.7	90	7.0	3.1
S e m	0.1	0.4	0.4	9	0.3	8.8	0.7	0.5
6 hrs	4.0	20.3	1.1	68	4.0	63	7.6	4.4
S e m	0.2	0.7	0.3	6	0.5	12	0.3	0.5
24 hrs	5.0	19.1	1.4	75	5.7	127	5.6	4.8
S e m	0.5	0.4	0.1	5	0.3	15	0.6	0.2
Weight control	2.9	28.6	2.6	121	6.7	175	6.6	4.3
S e m	0.2	1.5	0.2	14	0.4	17	0.6	0.2
High riboflavin	9.8	26.2	3.6	226	8.1	198	7.1	6.0
S e m	0.8	0.6	0.1	20	0.4	11	0.7	0.2
Kidney								
	mg							
Deficient control	333	16.3	4.9		11.4	1271	11.4	1.73
S e m	8	0.5	0.9		1.5	106	1.3	0.02
After dose 0.5 hr	331	17.5	13.7		14.2	1532	12.4	1.77
S e m	12	0.6	1.9		1.2	115	0.9	0.04
2 hrs	338	18.9	6.6		11.2	1436	13.3	1.74
S e m	17	0.4	0.6		1.4	96	1.0	0.07
6 hrs	356	17.9	5.2		13.5	1309	12.6	1.81
S e m	14	0.7	0.7		1.6	91	1.1	0.02
24 hrs	366	19.1	7.8		13.1	1626	14.0	2.01
S e m	30	0.4	0.4		1.6	123	0.8	0.03
Weight control	317	21.2	7.7		11.0	1589	12.7	2.41
S e m	14	0.4	0.5		1.6	115	0.7	0.1
High riboflavin	784	20.0	14.9		17.5	2155	22.8	2.69
S e m	52	0.6	0.6		1.0	123	1.2	0.08

TABLE III—*Concluded*

S e m = the standard error of the mean

\* The average initial weights of the deficient, weight control, and high riboflavin groups were 44, 43, and 45 gm, respectively, the gains in weight per rat per day were 0.79, 0.81, and 4.79 gm, and the food consumed per gm of weight gain was 5.4, 4.8, and 3.0

† Duplicate determinations for free riboflavin on tissues from one rat of each group showed that a large amount of free riboflavin was present in liver after 0.5 hour, 3.69  $\gamma$  per gm, 2 hours, 0.66, and 6 hours, 0.65, in kidney, after 0.5 hour, 7.8  $\gamma$  per gm, 2 hours, 0.54, and 6 hours, 0.92

‡ Glycine oxidase samples were incubated at pH 8.3, which is below the optimum

growth gave tissue coenzyme levels at 23 days which were but little higher than those of completely deficient animals (Table I). More total flavin was present, of course, because of the larger size. Weight control animals had significantly lower FMN levels in liver, kidney, and heart than the controls on a full diet, although there was no appreciable difference in FAD (Tables I and III).

*Protein*—Differences observed in tissue enzyme or coenzyme levels were uncomplicated by changes in protein concentrations, since changes of significance were not observed among the various experimental groups (Table I).

*Enzymes*—*DPNH dehydrogenase* activities were remarkably stable in spite of prolonged omission of riboflavin from the diet. At 3 weeks none of the deficient tissues tested was demonstrably lower in this enzyme activity. Restriction of food intake was also without demonstrable effect. By 12 weeks a slight fall in tissue levels was apparent, particularly when measured with ferricyanide as electron acceptor. This acceptor would be expected to measure most nearly the DPNH dehydrogenase itself, rather than intermediate links in the electron transport chain (12). Not all of the differences were statistically significant, but as a whole a trend downward is unmistakable.

After 23 days depletion, the *xanthine oxidase* level of riboflavin-deficient liver had dropped to 63 per cent of that of the weight control, but other tissues failed to show any significant change (Table I). Since previous investigators (13), using manometric techniques and other methods of measurement, have reported that the xanthine oxidase of liver falls to 25 per cent or less in riboflavin depletion, longer depletion periods were induced. After 6 and 12 to 13 weeks without riboflavin, the hepatic xanthine oxidase was 35 per cent of that in litter mate controls (Fig. 1, Table III). Renal xanthine oxidase was less affected (Table III).

Hepatic *D-amino acid oxidase* of rats on a deficient diet 23 days fell to 34 per cent of the weight control animals, but the renal enzyme dropped

to only 80 per cent. In the partially deficient groups, a similar discrepancy between the changes in hepatic and renal D-amino acid oxidase occurred. Further depletion for nearly 8 weeks resulted in a hepatic level 20 per cent of that of well nourished animals.

Hepatic *glycolic acid oxidase* activity declined to 10 per cent of normal by 6 weeks of depletion, and only 1 or 2 per cent was left after 12 weeks. After 6 weeks depletion 20 per cent of the initial *glycine oxidase* and 70 per cent of *L-amino acid oxidase* remained in liver. In kidney, neither of

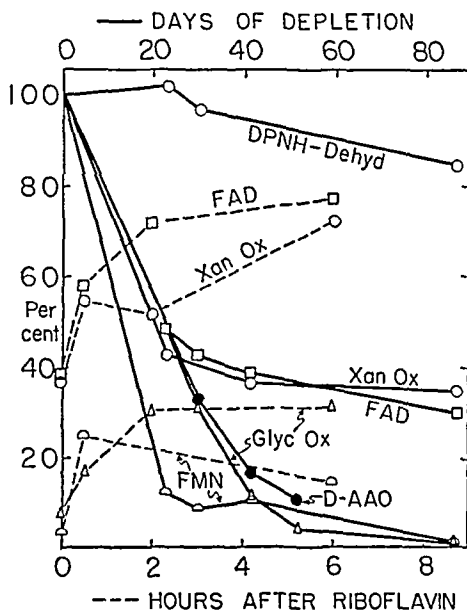


FIG 1 Depletion of hepatic enzymes and coenzymes during riboflavin deficiency and regeneration after injection of riboflavin. The values are all expressed as per cent of those found with a high riboflavin diet. The enzymes represented are DPNH dehydrogenase measured with cytochrome *c* (DPNH-Dehyd), xanthine oxidase (Xan Ox), D-amino acid oxidase (D-AAO), and glycolic acid oxidase (Glyc Ox). The regeneration data are calculated from Table III.

these last two enzymes was demonstrably lower after 6 weeks of deficiency than after a like period of restricted food intake (Table III).

*Weight control* animals experienced some loss in enzyme activity in comparison to the high riboflavin group (Tables I and III). DPNH dehydrogenase remained the same in both groups, but other enzymes were lower in weight controls. For example, in liver after 7 weeks D-amino acid oxidase was 88, L-amino acid oxidase 93, glycine oxidase 83, xanthine oxidase 72, and glycolic acid oxidase 53 per cent of the high riboflavin group. The effect of food and protein restriction in depressing xanthine oxidase has been repeatedly observed by others. Glycolic acid oxidase has been

shown to be even more sensitive to protein restriction<sup>3</sup> In the kidney, food restriction had almost as great an effect on the three amino acid oxidases measured as did riboflavin deficiency Conceivably, these changes reflect the decrease in substrates that would result from the lowered protein intake The decrease in certain enzyme activities with food restriction is mirrored by a decrease in FMN but not in FAD (Tables I and III)

*Realimentation*—Rates of regeneration of the coenzymes and several flavin enzymes in depleted rats were measured, following a dose of 600  $\gamma$  of riboflavin (Table III) The FAD increased rapidly, so that within 2 hours it was back to two-thirds of the control levels in liver and 95 per cent of normal in kidney FMN is slower to recover in both liver and kidney The high level of FMN plus free riboflavin at the 30 minute interval represents an unusual situation in which liver and kidney have a large amount of free riboflavin The content of free riboflavin in the tissues is normally very low By 6 hours after injection the free riboflavin had virtually disappeared, and, after 24 hours, FMN was still only 50 per cent or less of normal

Hepatic *glycolic acid oxidase* exhibited a rapid rise, doubling in 30 minutes and again in 2 hours, after which it remained almost stationary for the next two periods at about 60 per cent of the weight control level and only about one-third of the high riboflavin level FMN, the coenzyme of glycolic acid oxidase, after 24 hours, was likewise only about a third of the level in the high riboflavin group

Hepatic *D-amino acid oxidase* increased rapidly in 30 minutes but decreased in the later intervals, possibly owing to a shift of the FAD to other enzymes At the 24 hour period, due to a second injection of riboflavin at 12 hours, the level rose to 70 per cent of the weight control *Glycine oxidase* showed an increase almost to the weight control level by 24 hours Hepatic *xanthine oxidase* returned to the weight control level within 6 hours and to 80 per cent of the high riboflavin level in 24 hours Xanthine oxidase followed closely the FAD rise (Fig 1)

Changes in the kidney enzymes were less dramatic since the initial decreases were less than in liver *D-Amino acid oxidase*, which had fallen to only 50 per cent of the control level, returned to 70 per cent at the 30 minute interval Little additional change occurred at 24 hours *Glycine oxidase*, *L-amino acid oxidase*, and *xanthine oxidase* showed a slow increase during 24 hours

Livers and kidneys of weight control rats were smaller than those of depleted animals of equal body weight This finding suggests utilization of the limited food available to weight control animals for building other

<sup>3</sup> Data of R A Pesch and J H Clark, now partially published (*J Pharmacol and Exp Therap*, 117, 202 (1956), *Proc Soc Exp Biol and Med*, 91, 510 (1956))

tissues at the expense of these organs. The liver of deficient animals weighed progressively more following the injection of riboflavin, until, at the 24 hour interval, the average weight was 30 per cent above that of the deficient group. Since the protein concentration did not change significantly, the increase is due to growth of liver tissue. The total quantity of the enzymes in the whole liver, therefore, increased more than is revealed by the activity per unit weight.

*Enzyme Regeneration in Vitro*—Addition of FMN to deficient rat liver homogenates during enzyme assay resulted in an increase in the observed

TABLE IV  
*Effect of Coenzyme Addition during Measurement of D-Amino  
Acid or Glycolic Acid Oxidase*

Each value is the average of several analyses from the tissues of four or five rats. Activities are expressed as millimoles per kilo per hour.

Addition*	Glycolic acid oxidase				D-Amino acid oxidase			
	Liver				Liver		Kidney	
	None	Riboflavin	FMN	FAD	None	FAD	None	FAD
High riboflavin	225	224	235	242	135	154	1543	1711
S e m	12.3	11.0	12.7	14.0	10	14	51	43
Deficient, 7 wks	6	7	27	17	17.4	24.8	1147	1227
S e m	1.7	1.3	2.5	1.5	2.0	2.4	70	105
Deficient, 9-13 wks	3	3	17	12				
S e m	0.7	0.9	1.5	1.5				

S e m = the standard error of the mean

\* 6  $\gamma$  of riboflavin per ml of substrate or equimolar amounts of FMN or FAD were added for the glycolic acid oxidase measurements. 3.2  $\gamma$  of FAD per ml were added for the D-amino acid oxidase.

activity of glycolic acid oxidase (Table IV), indicating the presence of a certain amount of apoenzyme. However, even with FMN addition the activity was far below normal. The activity was only a little greater if homogenates were preincubated with FMN, even for long periods, prior to enzyme assay. The values found indicate that, after 9 to 13 weeks of depletion, less than 2 per cent of the original enzyme is present in active form and only 6 per cent is present as the apoenzyme. Thus 92 per cent of the original enzyme is no longer demonstrable. Added riboflavin did not increase the activity of glycolic acid oxidase. The activity of FAD is attributed to the presence of a small amount of FMN in the FAD preparation plus the FMN liberated enzymatically by the tissue.

In an analogous way, there was found to be only a limited restoration

of D-amino acid oxidase activity upon the addition of the coenzyme, FAD, to deficient homogenates (Table IV) The data indicate that in the liver, after 7 weeks deficiency, about 11 per cent of the original enzyme remains in active form and only about 5 per cent is present as the apoenzyme, whereas about 84 per cent is no longer demonstrable Similarly in the

TABLE V  
*Regeneration of Glycolic Acid Oxidase in Liver Slices  
from Riboflavin-Deficient Rats*

Activities are expressed in millimoles per kilo wet weight, per hour Each value is the average of four to eight determinations except as noted All incubations were for 1 hour

Group		FMN	FAD	Protein	Glycolic acid oxidase		DAO*
					No FMN added to substrate	With FMN added to substrate	
		$\gamma$ per gm	$\gamma$ per gm	mg per gm			
Normal	Homogenate not incubated	3 50	28 5	170	222	229	112
	S e m	0 53	0 60	0 8	20	20	
	Slices incubated in rat serum	4 80	20 8	140	164		90
	S e m	0 14	0 13	0 8	21		
	Slices incubated in rat serum + 6 $\gamma$ FMN per ml	10 80	17 2	134	160		84†
	S e m	0 03	0 10	0 7	12		
Deficient	Homogenate not incubated	0 19	7 29	190	4 6	15 3	21
	S e m	0 02	0 24	0 1	0 01	0 05	
	Slices incubated in rat serum	0 50	4 95	145	9 2	15 1	11
	S e m	0 01	0 21	0 1	0 11	0 15	
	Slices incubated in rat serum + 6 $\gamma$ FMN per ml	8 21	5 98	143	21 3	25 1	11†
	S e m	0 63	0 40	0 8	1 0	0 5	

S e m = the standard error of the mean

\* These are average values for D-amino acid oxidase on two samples of liver slices

† These slices were incubated in rat serum plus 19  $\gamma$  of FAD per ml

kidney there is little apoenzyme shown to be present, although the total enzyme loss is much less than in liver

In deficient liver slices, regeneration of glycolic acid oxidase was demonstrated to a limited extent (Table V) after incubation of the slices in rat serum with added FMN The level attained was only one-tenth that in normal rat liver A variety of incubation media with different salt mixtures was tried without inducing greater regeneration of the enzyme activity Slices incubated without FMN addition showed a decrease in FAD



and an increase in FMN, with some flavin leaking from the tissue into the serum. The protein of both normal and deficient slices decreased 25 per cent during incubation, which accounts for the fall in enzyme activity of normal slices.

D-Amino acid oxidase in deficient liver slices showed no evidence of regeneration upon incubation with FAD or FMN (not shown). Nor was there any means found to increase the FAD of liver slices through incubation with riboflavin or FMN.

#### DISCUSSION

The values reported here for D-amino acid oxidase are of an order of magnitude higher than most of the values in the literature<sup>4</sup>. The average normal renal value is 20 times that reported by Hawkins (15) and 6 times that of Axelrod *et al* (16). The normal hepatic levels are 5 to 12 times those found by previous investigators (15–18). These discrepancies may help to explain the different results upon addition of FAD *in vitro*. Rossiter (17) found that FAD added to deficient tissue during incubation raised D-amino acid oxidase back to normal, which led to the conclusion that the apoenzyme was still present undiminished. The present study indicates that the apoenzyme as well as the FAD disappears in riboflavin deficiency. The discrepancy may result from analytical difficulties with older methods. The low values suggest that something other than the enzyme itself may have been limiting. FAD is rapidly destroyed in homogenates of kidney and liver. The measurement of oxygen consumption may have reflected the amount of FAD present rather than the amount of D-amino acid oxidase available. The findings presented indicate that, in the case of at least two flavin enzymes, both the protein and the prosthetic group disappear in riboflavin deficiency. This is of some theoretical interest. It would indicate that either the apoenzymes are less stable in the cell than the holoenzymes or that the rate of synthesis of the protein part of the enzymes is diminished in the absence of the prosthetic groups. The latter might imply a type of adaptive process concerned with synthesis of these flavoproteins.

Attempts to induce regeneration of glycolic acid oxidase, D-amino acid oxidase, or FAD *in vitro* were rather abortive, and suggest that suitable conditions were not discovered since regeneration *in vivo* is quite rapid.

Glycolic acid oxidase of mammalian tissue has been studied very little. Dohan (19) reported its presence in liver of rats and rabbits. Kun *et al* (20) purified it from rat liver and showed its coenzyme to be FMN.

Ratner *et al* (21) were unable to obtain glycine oxidase from rat kidney,

<sup>4</sup> Umbreit and Tonházy (14), however, using methionine as the substrate, found activities for D-amino acid oxidase of normal rat liver about 2.5 times the present values obtained with D-alanine.

although they demonstrated its presence in kidneys of other species Krebs (22) had found earlier that rat kidney slices slowly deaminated glycine but that extracts were inactive. The use of less sensitive manometric methods, requiring longer incubation and more concentrated tissue preparations, may explain earlier difficulties in demonstrating the presence of this enzyme. Rat kidney is twice as rich as liver in this enzyme, but so far tests for its presence in other tissues have been negative. L-Amino acid oxidase is also much higher in rat kidney than in liver.

The decrease in xanthine oxidase in deficiency is less than that described by other investigators (13). Inclusion of molybdenum in the diet and the specificity and sensitivity of the analytical method used may account for the differences.

One of the most striking findings is the range in sensitivity to deficiency among the various flavin enzymes. Each enzyme decreases in activity at a different rate and to a different degree with the progression of deficiency, so that the enzymatic pattern changes to a considerable extent with time. The order of sensitivity to deficiency in liver, with the per cent of original activity remaining after 6 weeks, is glycolate oxidase 8, D-amino acid oxidase 17, glycine oxidase 21, xanthine oxidase 33, L-amino acid oxidase 67, and DPNH dehydrogenase over 90. One might anticipate that the enzymes with more readily dissociable prosthetic groups would be the first to suffer. It is true that the first three enzymes listed have all been reversibly dissociated from their prosthetic groups, whereas the last three have not. Nevertheless, in the kidney, glycine oxidase and L-amino acid oxidase are about equally affected by deficiency, although only the first has been shown to be dissociable (23). It remains to be explained why the same enzyme activities are affected quite differently in different tissues by riboflavin deficiency. Thus nearly all renal enzyme activities measured are more resistant to deficiency than are the same activities in liver.

#### SUMMARY

1 Four tissues of rats on four different dietary régimes have been analyzed for two riboflavin coenzymes, FMN and FAD, and for six enzymes. Levels have been determined for animals receiving (a) a high riboflavin diet *ad libitum*, (b) the same diet in restricted quantity, (c) a riboflavin-free diet, and (d) a diet low in riboflavin.

2 Of the normal tissues, the liver was richest in FAD, in DPNH dehydrogenase, xanthine oxidase, and glycolic acid oxidase. The kidney was richest in FMN, in D- and L-amino acid oxidases, and in glycine oxidase. Brain was lowest in all these enzymes. It contains only a tenth as much DPNH dehydrogenase as liver, even though its oxygen consumption is at least as great.

3 Remarkable differences were found in the rates at which the coen-

zymes and enzymes fall during depletion. In the brain changes were negligible. In liver FMN was lost more rapidly than FAD and some enzymes showed rapid and large decreases. In kidney and heart changes were much smaller.

4 DPNH dehydrogenase had the highest activity in all tissues and showed no decrease in moderate deficiency. In severe deficiency it showed a downward trend.

5 Hepatic enzymes in order of their sensitivity to riboflavin deficiency are glycolate oxidase, D-amino acid oxidase, glycine oxidase, xanthine oxidase, L-amino acid oxidase, and DPNH dehydrogenase. Xanthine oxidase decrease was parallel to FAD in liver, but D-amino acid oxidase and glycolic acid oxidase decreases were parallel to FMN. Both the protein and coenzyme moieties of the last two enzymes disappear in depleted rats. The large decrease in these enzymes did not occur in weight control animals. Hence, dietary riboflavin had some special rôle in the formation or maintenance of these enzymes.

6 Rapid regeneration of FAD and of glycolic acid oxidase, D-amino acid oxidase, and xanthine oxidase in deficient liver has been demonstrated *in vivo*. FMN, L-amino acid oxidase, and glycine oxidase come back more slowly.

7 Regeneration of glycolate oxidase in liver slices has been shown to a limited extent. Under the same conditions D-amino acid oxidase did not regenerate.

8 Weight control animals on restricted food intake with ample riboflavin showed lower than normal activity for a number of enzymes in both liver and kidney.

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# STUDIES IN VITAMIN E DEFICIENCY

## II THE INFLUENCE OF ADRENOCORTICOTROPIN AND OF TOCOPHEROL COMPOUNDS ON ADRENAL ACTIVITY\*

By HARRIS ROSENKRANTZ

(From the Worcester Foundation for Experimental  
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(Received for publication, March 12, 1956)

In a previous paper (1) it was reported that an elevation in oxygen consumption occurred in the adrenal cortex of tocopherol-deprived rabbits. It was also mentioned that the adrenal gland from vitamin E-deficient rabbits had the capacity to respond to adrenocorticotrophic hormone (ACTH) with a further increase in oxygen uptake. That the oxygen consumption of adrenal glands is influenced by ACTH has been shown for the rat (2, 3), dog (4, 5), cattle (6, 7), hog (8), and guinea pig (9). Tissue preparations consisted of bisected adrenal glands or adrenal cortical slices<sup>1</sup>.

It appeared worth while to pursue a possible correlation between oxygen consumption and steroid production. Perhaps the increased activity of the adrenal in vitamin E deficiency was not only a reflection of a non-specific stress situation (1) but was also biochemically related to the tocopherol-depleted condition of the gland. It is known that the adrenal and pituitary contain the highest concentrations of vitamin E, as compared with all other tissues in the body (11). Furthermore, Brummel *et al* (12) have indicated that part of the increase in oxygen consumption due to ACTH is used for the C<sub>11</sub>-oxygenation of steroids. Obviously the oxygen actually introduced into the steroid molecule represented a minute quantity of the total oxygen consumption.

The present investigation was designed to compare the oxygen uptake and steroid production of adrenals *in vitro* from vitamin E-deprived and tocopherol-supplemented rabbits. In addition, the influence of tocopherol compounds on adrenal glands *in vitro* with reduced vitamin E content was studied. Sliced glands and adrenal homogenates with and without ACTH were tested. Other workers have demonstrated an effect *in vitro* of ACTH

\* This investigation was supported by a grant from the Muscular Dystrophy Associations of America, Inc.

<sup>1</sup> Stack-Dunne and Young (10) have indicated that stimulation of oxygen uptake in adrenal homogenate by ACTH had been accomplished. However, examination of the original papers referred to did not permit this interpretation and has been so acknowledged by Stack-Dunne in a personal communication. That ACTH may influence the respiration of adrenal homogenates was suggested by preliminary data from this laboratory, which was quoted (*Chem and Eng News*, 32, 3945 (1954)).



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on reducing substances in rat (13-15), cow (14, 16), dog (17), and frog (18) in adrenal preparations with intact cells

Contrary to the findings on the homogenate fraction from adrenals from rat (13-15), cow (14, 16), dog (17), and hog (19), it was observed that ACTH does elicit an elevation in the levels of reducing substances in rabbit adrenal homogenates. An increment in oxygen consumption paralleled the rise in reducing material.

The studies on tocopherol compounds also revealed a stimulation of reducing substance which was more closely associated with  $\alpha$ -tocopherylhydroquinone than  $\alpha$ -tocopherol or its quinone.

### *Methods*

White, New Zealand rabbits, housed in glass metabolism cages, were raised on a purified diet lacking in vitamin E. The methods for observing the course of the deficiency have been reported previously in detail (1). Rabbits initially weighing less than 1000 gm exhibited nutritional muscular dystrophy between 3 and 4 weeks, while heavier animals resisted the deficiency for 1 or 2 additional weeks.

The animals were sacrificed by stunning and the adrenals rapidly removed to a cold dish, where they were carefully trimmed and studied as bisected glands, quartered glands, cortical slices, whole gland homogenate, or cortical homogenate. Razor blades were used for halving or quartering the glands, while a Stadie microtome served in the preparation of slices. When quartering of the gland was performed, a quarter of an adrenal from each rabbit was introduced into a flask so that a piece of adrenal from each animal was represented in each incubation vessel (13, 20). Homogenates were formed in an all-glass, conical homogenizer of 1 ml capacity or a 5<sup>3</sup>/<sub>4</sub> ml Potter type glass homogenizer. No attempt was made to remove cell debris because of the small quantity of tissue available. Microscopic examinations of a few homogenate aliquots revealed few intact cells.

The preparation of homogenates and all incubations were carried out in a Krebs-Ringer-phosphate medium with or without 0.0009 M calcium at pH 7.32. The major substrate was 0.1 ml of 10 per cent glucose, which was tipped in with or without 0.1 ml of ACTH<sup>2</sup> (5 to 10 units of Astwood's oxycellulose preparation or Armour's standard preparation, LA-1-A) from the side arm of Warburg vessels after a 5 to 15 minute equilibration period. Bovine serum albumin or ACTH inactivated by the hydrogen peroxide procedure of Hofmann and Davison (15) served as control proteins.

<sup>2</sup> Our sincerest gratitude is extended to Dr. Astwood for samples of his ACTH, to Dr. Meyer for revealing the details of his tetrazolium modification before publication, to Dr. Dorfman for samples of steroids, to Dr. Saffran for his prepublication communication on the procedure for methylene dichloride purification.

When the available tissue was more than 30 mg, 3 ml of medium were usually employed, less than 30 mg of tissue were incubated in a 1 ml volume in 5 ml vessels. In most instances, the gaseous atmosphere was air.

Steroid production was determined by the modified blue tetrazolium reaction of Meyer and Lindberg (21)<sup>2</sup>. Calibration curves were recorded for corticosterone, cortisone, and hydrocortisone<sup>2</sup> by employing the following final concentrations for a final volume of 1 ml solution: 0.0045 N tetramethylammonium hydroxide, 0.025 per cent blue tetrazolium, and approximately 93 per cent ethanol, 20 minutes at 38° and immediately read at 530 m $\mu$ .

Steroid recoveries were investigated with ethyl acetate, chloroform, and methylene dichloride as solvents. The method of extraction finally selected was that of Saffian and Schally (20), in which specially purified methylene dichloride was employed<sup>2</sup>. The incubation fluid and a 1 ml rinsing of the respiration vessel were transferred to a 10 ml glass-stoppered tube which had been washed with methylene dichloride and dried with air before use. 4 ml of methylene dichloride were used to extract the aqueous phase, and a 1 ml aliquot of the organic layer was evaporated to dryness in a micro-Coleman tube under a stream of nitrogen, the colorimetric assay being applied to the residue. The residue from the remaining methylene dichloride solution was chromatographed in the benzene-aqueous methanol system of Bush (22) and the toluene- or ligroin-propylene glycol partitions of Zaffaroni and Burton (23) and Savard (24), respectively.

In the experiments with vitamin E compounds, *d*- $\alpha$ -tocopherylquinone and *d*- $\alpha$ -tocopherylhydroquinone were dissolved in propylene glycol (10 mg per ml), and 0.1 ml was tipped into the incubation vessel after the equilibration interval. An equal quantity of propylene glycol was introduced into the control flasks. In these studies, 200 mg per cent glucose were present in the medium and 100 per cent oxygen was the gas phase (25).

*d*- $\alpha$ -Tocopherylquinone was prepared by the method of Karrer and Geiger (26) and purified by the procedure of Tishler and Wendler (27). The tocopherylquinone was freshly hydrogenated over 5 per cent palladium on calcium carbonate to yield the corresponding hydroquinone, which was assayed by the ferric chloride-dipyridyl reagents at the moment incubation commenced.

### Results

It was desirable to eliminate the possibility that the ACTH was being utilized as a substrate and thus initiating variations in oxygen consumption. The use of bovine serum albumin as a control protein gave variable results and was discarded. Oxygen consumption in the presence of hy-

drogen peroxide-inactivated ACTH (15) corresponded to that of control adrenals. This observation, in addition to the inability of active ACTH to influence the respiration of adrenal medulla preparations, indicated that active ACTH was not functioning merely as a substrate for adrenal cortical incubations. Periods of equilibration were necessary after tipping in hydrogen peroxide-inactivated ACTH since trace quantities of hydrogen peroxide caused some evolution of gas. This was probably due to catalase activity since, in one experiment, inhibition of catalase with sodium sulfide (28) prevented the evolution of gas.

The oxygen consumption of bisected glands and adrenal cortical homog-

TABLE I  
*Effect of ACTH on Oxygen Consumption of Normal Rabbit Adrenal*

Cortical preparation	ACTH units per cortex	$Q_{O_2}$ , $\mu$ l per hr per mg wet weight $\pm$ average deviation (range)		Per cent increase
		Addition of bovine serum albumin or inactivated ACTH	Addition of ACTH	
Bisected	1250* (LA-1-A)	1.96 $\pm$ 0.54 (3)† (1.28-2.79)	2.50 $\pm$ 0.74 (2) (1.76-3.24)	28
Bisected	5‡ (Astwood)	0.74 $\pm$ 0.32 (8) (0.34-1.67)	1.11 $\pm$ 0.33 (8) (0.64-1.95)	50
Homogenate	5‡ (Astwood)	0.18 $\pm$ 0.02 (9) (0.08-0.27)	0.49 $\pm$ 0.16 (10) (0.18-1.07)	172

\* An equal weight of bovine serum albumin was used as a non-specific protein control.

† Number of animals studied.

‡ ACTH inactivated by hydrogen peroxide (15) was utilized as its own protein control.

enates in the presence and absence of ACTH is summarized in Table I. Adrenals from the same animal and among litter mates were compared. A 28 to 50 per cent increase in oxygen uptake was observed for bisected glands treated with ACTH. The peak activity of ACTH occurred at about 1 hour, as seen in Fig. 1. This is in agreement with findings on perfused (29, 30) and sliced (14) glands.

Similar results were obtained with adrenal cortical homogenates. Although absolute  $Q_{O_2}$  values were lower than those of bisected or sliced preparations, greater responses were seen in the presence of ACTH. Calcium (0.0009 M) additions, compatible with the phosphate buffer employed, showed little effect on the activity of the excess ACTH concentrations used.

The assumption that the ACTH stimulation of adrenal cortical respiration was related to steroidogenesis afforded an opportunity to collect evi-

dence for an ACTH effect on steroid production in rabbit adrenal homogenate. The difficulty in isolating the microquantities of steroids from individual rabbit adrenals directed attention to assay procedures related to certain structural features of the steroid molecule. The tetrazolium reagent which determines the  $\alpha$ -ketol side chain of steroids was selected for its sensitivity. In addition, Elliott *et al* (31) have demonstrated a close correlation between ultraviolet absorption near  $240\text{ m}\mu$ , characteristic

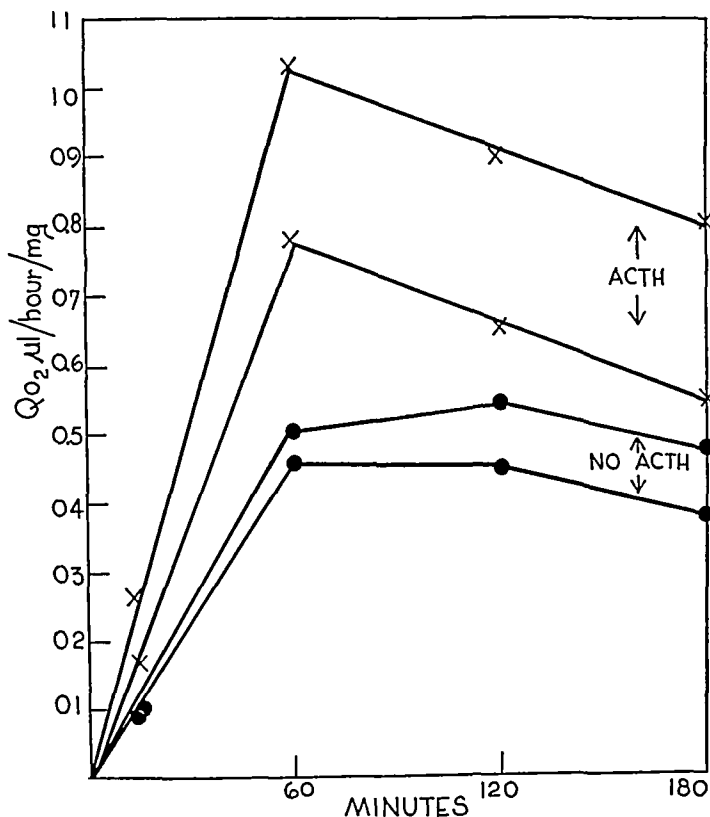


FIG 1 The peak activity of Astwood's ACTH on oxygen uptake of rabbit adrenal cortical preparations

for the  $\Delta^4$ -3-keto group of steroids, and the tetrazolium reaction. Moreover, Saffran *et al* (13) have established a parallelism between the absorption at  $240\text{ m}\mu$  by steroids and the eosinophil bioassay. Utilizing cortisone as a reference standard, these workers compared spectrophotometric analyses and bioassay and obtained a correlation coefficient,  $r = 0.936$ . In the present study it was recognized that corticosterone is the major active steroid released by rabbit adrenal and that the tetrazolium measurements represented both active and inactive  $\alpha$ -ketolic steroids.

It was confirmed that blue tetrazolium was found to be more sensitive

than triphenyltetrazolium chloride and 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride (31) Corticosterone, cortisone, and hydrocortisone gave equivalent densities (0.25 for 5  $\gamma$ ) and maximal color development

TABLE II  
*Influence of ACTH on Steroid Production by Rabbit Adrenal*

Diet of animal	No of animals in study	Adrenal preparation	ACTH*	BT material, $\gamma$ per 100 mg wet weight	Per cent increase	
					BT	O <sub>2</sub>
Stock pellet	3	Whole homogenate	—	42 (35-49)†		
			+	63 (38-88)	50	18
" "	8	Cortex homogenate	—	40 (13-68)		
			+	90 (67-167)	125	107
" "	6	Cortex homogenate	—	51 (Pooled)‡		
			+	71 "	39	20
" "	5	Cortex homogenate + 120 $\gamma$ corticosterone	—	113 "		
			+	150 "	32	
Purified + <i>d</i> - $\alpha$ -tocopherol	3	Bisected gland	—	27 "		
			+	40 "	50	29
" + " §	5	Whole homogenate	—	6 (2-9)		
			+	8 (5-16)	33	44
" + "	5	Whole homogenate	—	44 (20-72)		
			+	66 (47-87)	50	28
" + "	2	Cortex homogenate	—	22 (19, 26)		
			+	44 (39, 56)	100	70
" + "	3	Cortex homogenate	—	15 (Pooled)		
	3	Cortex homogenate	—	19 "		
	3	Cortex homogenate	+	37 "	118	120
Purified, no tocopherol	3	Bisected gland	—	48 "		
			+	121 "	150	
" " "	4	Cortex homogenate	—	69 "		
			+	95 "	38	100
" " " ¶	7	Whole homogenate	—	46 (29-55)		
			+	63 (61-78)	37	34
" " "	4	Whole homogenate	—	140 (41-369)		
			+	176 (63-379)	26	23

\* 10 units of Astwood's ACTH were used for maximal stimulation of each gland

† The numerals in parentheses give the range of values

‡ BT determinations were made on pooled glands

§ These rabbits were the litter mate controls for those discussed in the last footnote to this table

|| Hydrogen peroxide-inactivated ACTH

¶ Litter mates of controls mentioned under foot-note §

in 20 minutes under the conditions used with the blue tetrazolium reagent (BT) The density of the reagent and medium blanks was approximately 0.06 This BT method gave relatively low blanks, and recoveries above 90 per cent could be obtained with methylene dichloride as the extractant Both ethyl acetate and chloroform gave variable results, higher blanks, and recoveries between 70 and 90 per cent

Applications of the complete procedure of incubation, extraction, and colorimetric analysis to ovaries, skeletal muscle, diaphragm, liver, kidney, and intestinal homogenates and slices gave low BT values The BT content of these tissues was approximately equivalent to 13 and 1 per cent of

TABLE III  
*BT Levels in Total Adrenal Homogenate from Litter  
Mate Rabbits in Several Colonies*

No. of rabbits	Days on purified diet	Condition	Average urinary creatine  mg per 24 hrs	BT	
				Average  γ per 100 mg wet weight	Range  γ per 100 mg wet weight
3	17-24	Control	13	61	50-72
3	17-24	Deficient	44	220	128-379
1	20	Control	2	9	
3	20	Deficient	14	34	30-39
3	23-30	Control	10	17	6-26
6	24	Deficient	82	53	37-69
2	26	Control	7	8	7-8
3	26	Deficient	13	11	10-12
3	29-34	Control	10	7	2-9
3	26-28	Deficient, paralysis	95	66	34-99
3	32-38	Control	10	7	3-9
4	32-34	Deficient, paralysis	80	66	52-83

adrenal levels from control and from deficient animals, respectively Methylene dichloride extracts of solutions containing ACTH did not exceed reagent blank levels

The results of these BT determinations and the corresponding alterations in oxygen consumption for control and vitamin E-deficient rabbit adrenal are outlined in Table II An increase in BT material occurred in the presence of ACTH in bisected adrenals, whole gland homogenates, and adrenal cortical homogenates The increase ranged from 26 to 150 per cent, variations below 20 per cent were not considered significant Since it has been demonstrated that ACTH did not behave as a substrate, inactivated ACTH was not added to all control incubations in the BT studies

However, one illustration which includes inactivated ACTH is given in

Table II The tetrazolium measurements were performed with the older method of Chen and Tewell (32)<sup>3</sup> No difference existed between untreated and inactivated, ACTH-treated samples The group receiving the active ACTH clearly showed a rise in BT material

The increment in  $Q_{O_2}$  paralleled the elevation in BT levels Statistical analysis yielded a correlation coefficient,  $r = 0.897$ , and a  $t$  value 9.25 ( $p < 0.01$ ) The  $Q_{O_2}$  increases ranged from 18 to 120 per cent, and values above 15 per cent were significant Other workers obtained increments of 20 to 50 per cent for bisected or sliced adrenals from various species (2-8)

In one study, 120  $\gamma$  of corticosterone were added to the incubation me-

TABLE IV  
*Reactivity of Tocopherol Compounds with Blue Tetrazolium Reagent*

Substance	Concentration	Steroid equivalence	Reaction
	$\gamma$	$\gamma$	per cent
<i>d</i> - $\alpha$ -Tocopherol	25	3.9	16
	50	7.6	15
	75	11.1	15
	100	14.2	14
<i>d</i> - $\alpha$ -Tocopherylquinone	50	1.8	4
	100	3.6	4
	150	5.3	4
	200	7.1	4
<i>d</i> - $\alpha$ -Tocopherylhydroquinone	1	1.0	100
	2	2.1	95
	3	3.2	94
	5	5.4	93
	6	6.4	94

dium containing a normal adrenal cortical homogenate The ACTH effect did not appear to be inhibited by this steroid

It should be noticed (Table II) that BT values appear to vary greatly in comparable adrenal preparations However, BT levels are of similar magnitude when the control and deficient rabbits, respectively, from the same litter are compared

A comparison of adrenal BT levels from control and vitamin E-deficient litter mates from several colonies is shown in Table III Irrespective of the days on the purified diet or the overt symptoms of nutritional muscular dystrophy, an elevation of BT content was discerned in the adrenals of tocopherol-deprived rabbits Again the BT fluctuation from colony to colony was obvious

<sup>3</sup> Thanks are due to Morey Feinstein for some of the early tetrazolium determinations

Several attempts were made to chromatograph the methylene dichloride residues. The wet weight of a rabbit adrenal cortex is approximately 20 mg in an animal weighing 1500 gm. From individual analyses and from calculations from the data in Table II, it was realized that perhaps 2 to 10  $\gamma$  of BT material would be available for chromatography. It was not desirable to pool adrenal extracts from the vitamin E-deprived animals since even litter mates were not at the same level of nutritional dystrophy as indicated by the variations in creatinuria and muscular paralysis. However, some pooled adrenal extracts of control animals were subjected to

TABLE V  
*Recovery of Tocopherol Compounds\**

Substance	Quantity added	Ferric chloride-dipyridyl	Ultraviolet absorption	Recovery	Steroid BT equivalence	BT interference
	$\gamma$	$\gamma$	$\gamma$	per cent	$\gamma$	per cent
<i>d</i> - $\alpha$ -Tocopherol	1125				120	
	1125				120	
	1125		680†	60	108	16
<i>d</i> - $\alpha$ -Tocopherylquinone	100		75‡	75		
	1000		738	74	19	3
					15	21
<i>d</i> - $\alpha$ -Tocopherylhydroquinone	72				24	19
	128				40	26
	156	31		20	66	21
	314	57		18	63	20
	320				87	22
	390	73		19	72	17
	432	77		18	100	19
	540	122		23	120	19
	648				114	15
	780	96		12		

\* Each experiment was performed in duplicate

† Determined at 292  $m\mu$

‡ Determined at 262  $m\mu$

paper chromatographic fractionation in a benzene-aqueous methanol system. One such experiment yielded a paper chromatogram which contained a more intense BT zone, migrating at a rate similar to corticosterone, for the residue representing an ACTH-treated homogenate as compared with that of an untreated sample. Naturally additional studies are necessary to substantiate this finding.

The final phase of this work involved incubation of quartered adrenal glands and whole gland homogenates with tocopherol compounds. Rabbits on a stock and purified diet were studied. Vitamin E-deficient rabbits afforded test animals, the adrenal activity of which perhaps would be more sensitive to exogenous tocopherol compounds if such compounds



were associated with steroidogenesis. A crude estimate of tocopherol depletion of the adrenal gland could be obtained by colorimetric analysis. Residues from methylene dichloride extracts were subjected to ferric chloride oxidation in the presence of  $\alpha, \alpha'$ -dipyridyl (EE). Adrenal tissue from controls invariably initiated a positive reaction, while extracts from adrenals of vitamin E-deficient animals were negative.

The reactivity of tocopherol compounds with the BT reagents was also

TABLE VI  
*Influence of Tocopherol Compounds on Steroid Production in Quartered Adrenals from Vitamin E-Deficient Rabbits*

Substance	Concentration	Total BT	Correction for interference by tocopherol compounds*	BT	Increase
	$\mu\text{M}$	$\gamma$	$\gamma$	$\gamma$ per 100 mg dry weight	per cent
Control		13		37†	
		16		62 (50-75)‡	
<i>d</i> - $\alpha$ -Tocopherol	1 1	16	3	43	12
	2 3	37	6	92	48
	2 3	27	4	92	48
	2 3	30	5	123	98
<i>d</i> - $\alpha$ -Tocopherylquinone	1 1	12	1	35	0
	2 2	28	1	88	42
	2 2	28	1	120	93
	2 2	20	1	92	48
<i>d</i> - $\alpha$ -Tocopherylhydroquinone	1 6	64	13	205	465
	3 2	188	38	414	567
	3 2	114	23	364	488
	3 2	183	37	381	515

\* Correction percentages are as follows: 15 per cent for tocopherol, 4 per cent for tocopherylquinone, and 20 per cent for tocopherylhydroquinone.

† Control level for lower concentration of substrates.

‡ The values in parentheses give the range of control BT values for three determinations.

studied. This would permit a correction for their interference in the analytical method used. The results of the tetramethylammonium hydroxide-blue tetrazolium reaction with the tocopherol compounds are presented in Tables IV and V. Direct reaction of the substrates and reagents indicated that tocopherol will yield a BT steroid equivalence of 15 per cent, tocopherylquinone of 4 per cent, and tocopherylhydroquinone of 95 per cent.

When the substrates were carried through the complete incubation and extraction procedure, the extent of interference by tocopherol and its quinone is similar to that on direct reaction with BT (Table V). Ultraviolet

absorption indicates that approximately 60 per cent of the added tocopherol is extracted into methylene dichloride after one partition and that the recovered material gave a steroid equivalence of approximately 16 per cent. 75 per cent of the added tocopherylquinone is recovered and yields a steroid equivalence of approximately 4 per cent. Recoveries of tocopherylhydroquinone (THQ) varied somewhat, due to the unstable character of this molecule, and averaged 17 to 26 per cent by both the EE and BT chem-

TABLE VII

*Effect of Varying Concentrations of  $\alpha$ -Tocopherylhydroquinone on Adrenal Steroid Levels Corrected for Presence of  $\alpha$ -Tocopherylhydroquinone\**

Added THQ	Total BT	Total EE	Adrenal	Per cent increase
$\gamma$	$\gamma$	$\gamma$	$\gamma$ per 100 mg dry weight	
0	9	1	29	
90	14	5	31	6
450	38	13	110	280
900	53	24	115	296
0	7	1	25	
128	10	4	26	4
320	38	20	56	124
640	68	33	100	300
0	8	1	37	
142	13	4	41	11
355	35	10	119	222
710	72	44	137	270
0†	9	0	31	
77	13	1	38	22
386	35	10	95	223
777	56	24	139	350
0†	5	0	18	
95	15	6	29	60
237	28	14	44	145

\* Each determination was carried out in duplicate

† Results on vitamin E-deficient animals

ical assays. The same results were obtained in the absence and presence of boiled adrenal tissue. Because of its lability the quantity of THQ recovered was less after incubation and exposure to 100 per cent oxygen than when such treatment was omitted.

In the tissue incubations with vitamin E substrates it was decided to utilize quartered adrenals because of preparative ease and good reproducibility. The influence of tocopherol compounds on adrenal tissue in a Krebs-Ringer-phosphate-glucose medium in an atmosphere of 100 per cent oxygen is outlined in Table VI. Corrections have been applied for

the interference of the tocopherol substrates in the BT reaction, as described above. It can be seen that the tocopherol compounds have an influence on the BT content of adrenals. Highest activity was exhibited by THQ. The remarkable elevation in adrenal BT made it essential to determine the exact portion of the total BT which was originating from the substrates and steroids, respectively.

Specific colorimetry was an obvious approach, since THQ could be determined by both the EE and BT procedures while the  $\alpha$ -ketolic steroids were susceptible to the BT assay. It was found that corticosterone and cor-

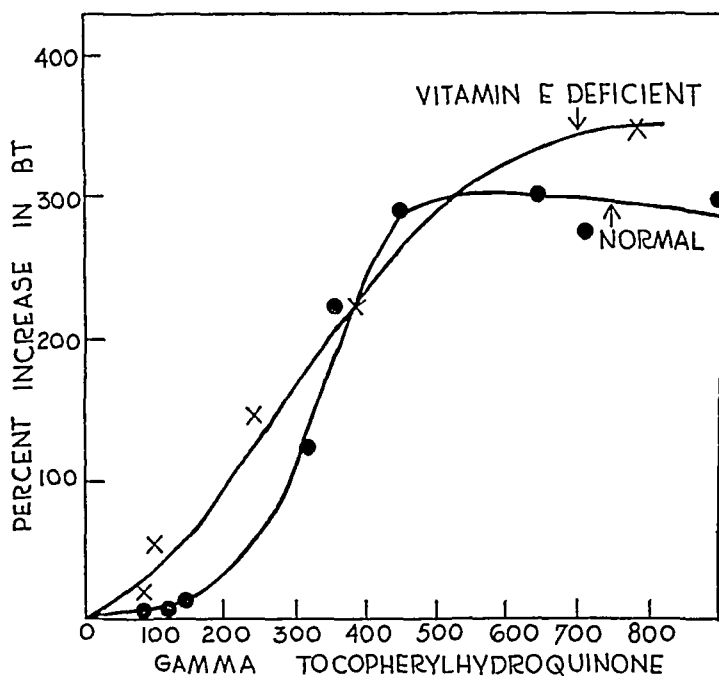


FIG. 2 The influence of varying doses of *d*- $\alpha$ -tocopherylhydroquinone on the per cent increase of BT material in quartered rabbit adrenal

tisone interfered in the EE test less than 2 per cent. Therefore, total BT would represent THQ plus steroid, and subtraction of the EE value would yield the steroid concentration. This type of correction was initiated in a THQ concentration study. Five individual experiments are presented in Table VII and in Fig. 2. The EE levels were subtracted from the total BT content before adjustment to a common reference point of micrograms per 100 mg. of dry weight of tissue. The EE concentration represented approximately 3 per cent of the added THQ. It would appear that living tissue increases the destruction of THQ since studies with boiled or no tissue indicated about 20 per cent recoveries. The results were in conformity with the profound influence of THQ on adrenal BT. A plot of per cent increase in BT *versus* concentration of THQ revealed an S-shaped

curve (Fig 2) THQ influence commenced near 150  $\gamma$  and reached a plateau at approximately 500  $\gamma$ . Adrenals from vitamin E-deficient rabbits may be influenced at lower concentrations of THQ, and the effect may continue above the plateau level of control animals.

Other approaches were undertaken to establish the individual donation of THQ and steroid in mixtures to total BT. Differential oxidation with ferric chloride destroyed all the THQ in a THQ-steroid mixture but too much steroid was also oxidized. A more gentle selective oxidation with molecular oxygen was promising but time-consuming and variable. No destruction of steroid occurred and 50 per cent of the THQ was oxidized in a half-hour interval.

Preferential partition into isooctane was not successful, although no steroid and significant quantities of THQ dissolved in isooctane. Methylene dichloride extracted approximately all of the steroid and as high as 50 per cent of the THQ.

Another approach was one of paper chromatography. In the toluene-propylene glycol system corticosterone migrated at a suitable rate for resolution from other corticosteroids. However, THQ migrated similarly to corticosterone, 19 to 25 cm in 20 hours, and therefore this system of chromatography could not be used. In the ligroin-propylene glycol system the steroids remained near the origin, while THQ moved 21 to 26 cm in 8 hours. The zones were observed under the ultraviolet lamp and by the blue tetrazolum reaction. The necessary areas were eluted into methanol and examined in a Cary spectrophotometer. Manipulation during recovery of the tocopherylhydroquinone resulted in almost complete conversion of the hydroquinone to the corresponding quinone.

This procedure was applied to extracts of tissue incubations. Little quantitative difference could be detected spectrophotometrically between the material from the control steroid zone and that from the corresponding zone from an incubation to which were added 128  $\gamma$  of THQ. When the concentration of THQ was increased, there appeared to be an increase in the concentration of the steroid zone. Ultraviolet analysis of the THQ zones as the quinone accounted for approximately 90 per cent of the material determined by the EE reaction.

Another BT-reactive zone was observed on chromatograms of incubated extracts untreated with THQ, which migrated slightly more slowly than THQ. Elution and spectrophotometric analysis indicated the presence of approximately 4  $\gamma$  of BT material. Although the tocopherylquinone interfered with the quantitative determination of the corresponding zone from THQ-incubated extracts, an increase in this material was suggested since the tocopherylquinone minimum near 240  $m\mu$  appeared to be elevated. This was observed in four experiments and has been interpreted as an increase in material absorbing near 240  $m\mu$ .

## DISCUSSION

Although the evidence is in favor of an ACTH effect on adrenal homogenates, the isolation and identification of steroids in the BT material would afford unequivocal proof of such an influence. It would be desirable to find a more reproducible preparative procedure, since, in addition to the obvious biologic variation that was observed, results varied with a specific homogenizer used, the time of homogenization, how the animal was killed, how the tissue was handled, and probably with other unrecognized factors.

The ACTH influence on oxygen consumption and BT production occurred in approximately 75 per cent of the cases studied. Among the failures were situations in which BT content was increased without alteration in  $Q_{O_2}$  and *vice versa*. The difficulty in obtaining more consistent responses to ACTH was not inherent in the homogenate system, since negative experiments were recorded on slices too. A similar per cent response of beef adrenal slices to ACTH has been reported by Haynes *et al* (14), and variability in perfusion studies has also been observed (29, 30).

In order to reconcile the present positive results of ACTH on adrenal homogenates with lack of such demonstration by other investigators, an explanation lies in three factors: species difference, the principal use of Astwood's oxycellulose-purified ACTH, and the preparation of the tissue. Adrenal preparations from other species were usually subjected to centrifugation for separation of certain cellular fragments and debris. This was not done in the present instance. Although centrifugation afforded more certainty in the elimination of intact cells, perhaps necessary components for the ACTH system were also deleted.

That ACTH could have an effect on homogenate preparations is supported indirectly by several lines of evidence. Sourkes and Heneage (33) have shown that adrenal homogenates oxidize the Krebs cycle intermediates more rapidly than slices. Saffran and Bayliss (25) have demonstrated the necessity of oxygen for an ACTH effect, Brummel *et al* (12) have implicated 11-hydroxylation with the increased oxygen consumption due to ACTH, and recently Hayano *et al* (34) have proved that 11 $\beta$ -hydroxylase uses molecular oxygen for the incorporation of the 11-hydroxyl group into the steroid molecule. That the necessary mechanisms for utilizing oxygen in the synthesis of steroids are available in adrenal homogenates seems clear. Several workers have demonstrated biosynthesis of corticosteroids in cell-free adrenal preparations (16, 18, 35), and ACTH has been assigned a regulatory rôle for one of these syntheses, the transformation of cholesterol to pregnenolone (36), in the intact gland (37).

The best evidence in the present investigation which indicated that an ACTH influence was being exerted on rabbit adrenal homogenate was the parallelism between increase in oxygen consumption and elevation in

reducing material. Such parallelism also existed in vitamin E-deficient rabbits in which nutritional stress resulted in  $Q_{O_2}$  (1) and BT increments in the absence of exogenous ACTH. A similar observation has been made for scorbutic guinea pig in which adrenal respiration (9), urinary formaldehydic substances (38), and blood ACTH (39) were elevated.

The work on the tocopherol compounds indicated that the BT reaction could be useful for determining tocopherylhydroquinone in extracts, on paper chromatograms, and in the presence of tocopherol.

The finding that tocopherol compounds did influence the concentration of BT material elaborated by the adrenal gland could be important. Tocopherol has been implicated in several biological systems, but its rôle has usually been assigned to its antioxidant property. Tocopherylhydroquinone had the greatest influence and tocopherol and tocopherylquinone presumably had their effect because of the ease of oxidation of the phenol to the quinone and the reduction of the latter to the hydroquinone. The possible existence of an oxidation-reduction system which relates tocopherylhydroquinone and tocopherylquinone to steroid-BT production may throw new light on the functions of vitamin E. It is tempting to point out that here may exist a biochemical rôle for the relatively high concentrations of vitamin E in the adrenal gland. Furthermore, an association of vitamin E and steroids leads to the realization that a link is available to correlate the necessity of  $\alpha$ -tocopherol in the reproduction of lower Mammalia and the protection of normal pregnancy by certain steroid hormones. Progesterone substances have been found in the adrenal (37, 40).

Another observation made on adrenal tissue exposed to tocopherol compounds was concerned with the reduction of tocopherylquinone to tocopherylhydroquinone. Preliminary experiments indicated that the hydrogen transfer was enzymatic since boiled adrenal tissue failed to show BT increments. It was found that ascorbic acid could act as a hydrogen donor in the presence of viable adrenal tissue and that reduced glutathione functioned to a lesser degree in this respect. Future studies will bring closer examination of this mechanism.

The skilful technical assistance of Miss Mary P. Kane and Mr. Judson Spalding is gratefully acknowledged.

#### SUMMARY

1. The influence of adrenocorticotrophic hormone (ACTH) and tocopherol compounds on adrenal preparations from control and vitamin E-deficient rabbits was investigated.

2. It was found that adrenal-reducing substances increased during vitamin E deprivation similarly to oxygen consumption. ACTH could also

elevate  $Q_{O_2}$  and blue tetrazolium (BT) materials in adrenal slices or homogenates

3 Tocopherylhydroquinone had a profound effect on BT levels in adrenal tissue, with less of an influence being exerted by tocopherol and tocopherylquinone

4 The implications of these findings in relation to nutritional stress and vitamin E function in reproduction have been discussed

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# THE INHIBITION OF ADENOSINE DEAMINASE BY 8-AZAGUANINE IN VITRO\*

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The biochemical mechanism underlying the cytotoxic and carcinostatic (1-3) effects of 8-azaguanine is still uncertain. Current hypotheses are that the compound acts directly as a metabolite antagonist to guanine utilization (4) or that it is incorporated into nucleic acids and that these fraudulent nucleic acids serve as the active pharmacological agents (5-7). An alternative hypothesis being explored in this laboratory postulates that 8-azaguanine, as a purine analogue, may inhibit certain of the enzymes related to purine metabolism. The present report is concerned with the observation that 8-azaguanine is a potent inhibitor of adenosine deaminase *in vitro* in both homogenate and isolated enzyme systems, the mechanism underlying this inhibition has been investigated.

## Methods

Inhibition of adenosine deaminase activity of tumor homogenates was assessed in the following manner. Frozen or chilled freshly excised adenocarcinoma 755 was homogenized in cold 0.2 M tris(hydroxymethyl)-aminomethane (Tris) buffer, pH 7.0, with an all-glass Potter-Elvehjem homogenizer. Duplicate 50 ml Erlenmeyer flasks were prepared which contained 1.0 ml of homogenate in the absence and the presence of 0.3 ml of  $5.6 \times 10^{-3}$  M adenosine. Inhibitors were introduced into the reaction mixture as indicated in the text. Sufficient 0.2 M Tris buffer was added to bring the total volume to 15.0 ml, and the reaction mixture was incubated with shaking in a Dubnoff apparatus for 1 or 2 hours at 37°. Enzymatic activities were halted by the addition of 1.0 ml of 50 per cent trichloroacetic acid. The amount of deamination that had occurred during the incubation was determined by measuring the amount of ammonia released, by adding 2 ml of saturated potassium carbonate solution to each flask.

\* An abstract of this paper has been published (Feigelson, P., Wu, M. S. L., and Davidson, J. D., Abstracts, American Chemical Society, 128th meeting, Minneapolis, 5C, Sept (1955)).

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The ammonia thereby liberated was transferred by aeration to collecting tubes containing 5.0 ml of 0.02 N sulfuric acid. The distilled ammonia was nesslerized and the optical density at 420 m $\mu$  was measured (8, 9). The difference between the endogenous ammonia formation in control flasks and the ammonia formed in the flasks containing adenosine was taken as indicating the adenosine deaminase activity of the homogenate. This activity was shown to be a linear function of the amount of tissue present.

For studies designed to gain insight into the intimate mechanisms underlying the inhibition of adenosine deaminase by 8-azaguanine, the homogenate system proved inadequate. Not only was there considerable endogenous ammonia formation but the presence of guanase in the homogenate was undesirable from two viewpoints. 8-Azaguanine was slowly deaminated by guanase (10, 11), thereby leading to a steady fall in the concentration of this inhibitor as the incubation period progressed. The resultant ammonia evolution still further increased the blank to be subtracted from the substrate-containing flasks. Attention was therefore directed towards the preparation of a soluble adenosine deaminase, largely free of guanase, which could be used in the sensitive and accurate spectrophotometric assay of Kalckar (12).

Acetone powder of rabbit intestinal mucosa, a rich source of adenosine deaminase (13), was prepared by conventional techniques. Since adenosine deaminase is soluble in distilled water (14), 0.5 gm of acetone powder was extracted three times with 10 ml of distilled water at 0°. These extracts were pooled and stored in a freezer. On being thawed at 4°, any precipitate was removed by centrifugation. Since this supernatant solution possessed high adenosine deaminase activity and only traces of 8-azaguanine deaminase, it was used without further purification.

The adenosine deaminase activity was assayed at room temperature with a Cary model No. 11 recording spectrophotometer. The enzyme was added in 0.1 ml as the last component of a mixture containing 0.5 ml of 10.5 or 20 mg per cent adenosine solution, various amounts of 8-azaguanine, and sufficient 0.1 M sodium phosphate buffer, pH 7.0, to yield a total volume of 2.8 ml in each cuvette. The enzyme was omitted in the controls. After mixing the solution in each cuvette, its optical density at 265 m $\mu$  ( $E_{265}$ ) was recorded continuously for 5 minutes. The enzyme-catalyzed conversion of adenosine to inosine resulted in a fall in  $E_{265}$  (12). The slopes of the initial linear declines were determined from the recordings. The initial rates of fall in  $E_{265}$  were shown to be a linear function of the amount of enzyme added, with 0.1 ml of the adenosine deaminase preparation producing a decrease in  $E_{265}$  of 0.238 per minute.

## Results

Table I depicts the results of an experiment in which various purine analogues were compared at isomolar concentrations as inhibitors of the

TABLE I  
*Effect of Purine Analogues on Adenosine Deaminase  
Activity in Vitro of Adenocarcinoma 755*

Analogue $3.3 \times 10^{-3}$ M	Adenosine deaminase activity	
	$\mu$ moles of $\text{NH}_3$ per hr per gm tissue	Per cent inhibition
None	13.7	
8-Azaguanine	0	100
8-Azaxanthine	9.5	31
2,6-Diaminopurine	9.9	28
6-Mercaptopurine	12.2	11

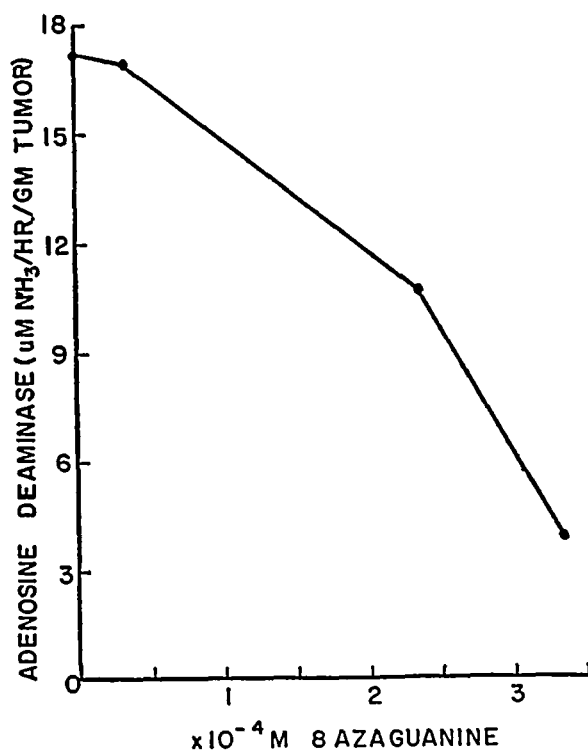


FIG 1 The inhibition of adenocarcinoma 755 adenosine deaminase by 8-azaguanine

adenosine deaminase of adenocarcinoma 755 homogenates *in vitro*. In these assays of ammonia evolution,  $3.3 \times 10^{-3}$  M 8-azaguanine completely

inhibited adenosine deaminase activity, whereas its normal catabolite, 8-azaxanthine (15, 16), was a much weaker inhibitor, 6-mercaptopurine and 2, 6-diaminopurine acted as still less efficient inhibitors. When lower concentrations of 8-azaguanine were tested, it was found that  $10^{-4}$  M levels of the compound still gave effective inhibition. Fig 1 shows the mean results of two such experiments in which 50 per cent inhibition of tumor

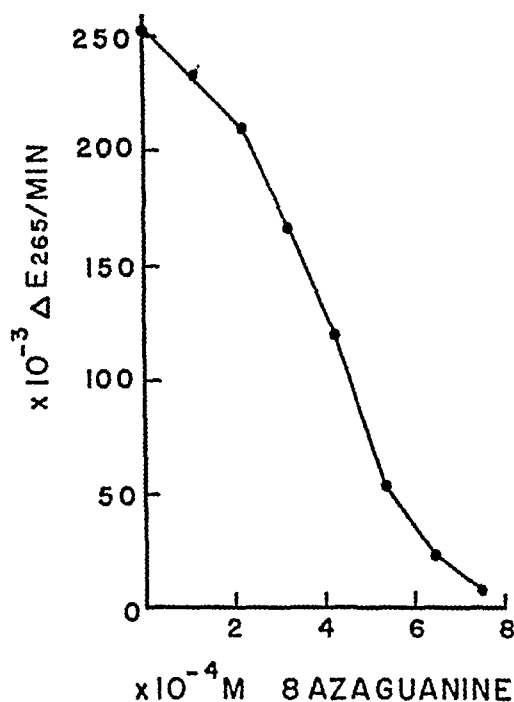


FIG 2 The inhibition of intestinal mucosal adenosine deaminase of rabbit by 8-azaguanine. To control and experimental spectrophotometer cuvettes were added 1.0 ml of  $3.75 \times 10^{-4}$  M adenosine, the indicated amounts of 8-azaguanine as 0.003 M solution, and 0.1 M sodium phosphate buffer (pH 7.0) to make the total volumes to 2.8 ml. To the experimental cuvette, 0.10 ml of adenosine deaminase was added, immediately mixed, and the change in  $E_{265}$  with time continuously recorded with the control cuvette serving as the blank. ● = the means of duplicate determinations.

adenosine deaminase activity was accomplished at  $2.6 \times 10^{-4}$  M 8-azaguanine. In Fig 2 are presented the results of similar studies made with the intestinal mucosal preparation of adenosine deaminase by the spectrophotometric assay system. The inhibition is manifested equally well in this system, although a slightly higher concentration of 8-azaguanine,  $4.1 \times 10^{-4}$  M, seems necessary for 50 per cent enzyme inhibition. The inhibition curve is not of simple exponential nature but is of the sigmoid type.

To determine whether 8-azaguanine is a competitive or non-competitive

inhibitor of adenosine deaminase, Lineweaver-Burk experiments were undertaken in which the enzyme activity was measured in the absence and presence of  $2.1 \times 10^{-4}$  M 8-azaguanine over a wide range of substrate concentrations. When the reciprocal of the enzyme activity is plotted against the reciprocal of the substrate concentration, curves are obtained, in the presence of and without 8-azaguanine, as indicated in Fig. 3. The upward

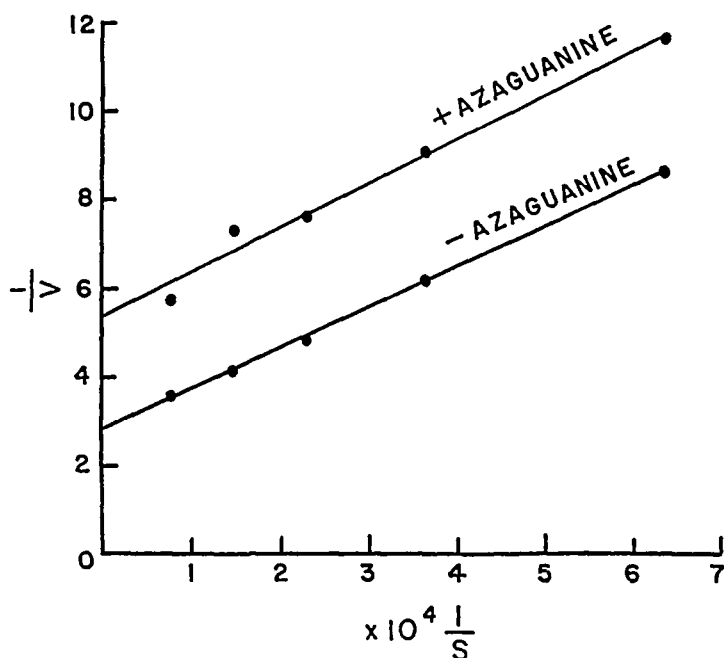


FIG. 3. The non-competitive inhibition of adenosine deaminase by 8-azaguanine. To control and experimental cuvettes were added the indicated concentrations of adenosine and sufficient 0.1 M sodium phosphate buffer (pH 7.0) to make the total reaction volume to 2.8 ml. To the experimental cuvettes was added 0.1 ml of adenosine deaminase. They were then immediately mixed and the changes in  $E_{265}$  recorded, with the control cuvette serving as the blank. Similar determinations were made in the presence of 0.10 ml of  $0.010$  M 8-azaguanine (upper line). ● = the means of duplicate determinations.

displacement of the intercept of the upper, 8-azaguanine curve points to a non-competitive inhibition with regard to the substrate (17). The calculated dissociation constants of the enzyme-substrate and enzyme-inhibitor complexes are  $K_s = 3.6 \times 10^{-5}$  and  $K_i = 2.8 \times 10^{-4}$ . Although valid for these particular experimental conditions, this  $K_i$  is not a true constant but varies with the inhibitor concentration, as will be seen.

Simple non-competitive inhibitors obey the relationship,  $\log(v/v_i - 1) = r \log I + 1/K_i$ , where  $v$  and  $v_i$  are the activities in the absence and presence, respectively, of the inhibitor at concentration  $I$ , and  $r$  is the number

of moles of inhibitor combining per mole of active enzyme center (17). However, when adenosine deaminase activity was determined in the absence and presence of increasing azaguanine concentrations and the values of  $\log (v/v_i - 1)$  were plotted against the log of the azaguanine concentration, an exponential curve was obtained (Fig 4) rather than a straight line with a constant slope of  $r$ . Since the slope increases with rising inhibitor concentration, the number of moles of 8-azaguanine which combine

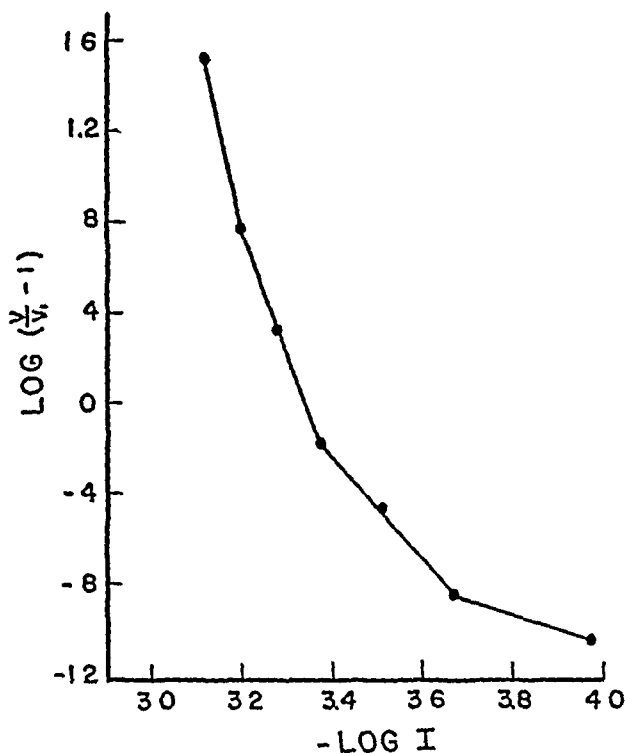


FIG 4 The determination of the number of moles of 8-azaguanine which combine per mole of adenosine deaminase. Experimental conditions are identical with those of Fig 2. The depicted curve represents the mean data of duplicate experiments.

per mole of enzyme likewise increases with rising 8-azaguanine concentration. By employing tangents to the curve, the following estimates of  $r$  and  $K_i$  can be made: at  $1 \times 10^{-4}$  M 8-azaguanine  $r = 1$ , and  $K_i = 9 \times 10^{-4}$ ; at  $7 \times 10^{-4}$  M 8-azaguanine  $r = 11$ , and  $K_i = 6 \times 10^{-25}$ . Therefore, for the interaction between adenosine deaminase and 8-azaguanine neither  $r$  nor  $K_i$  is a true constant but rather varies widely with the 8-azaguanine concentration.

To ascertain whether the 8-azaguanine-adenosine deaminase complex once formed was dissociable or whether 8-azaguanine formed an irreversible complex with the enzyme, an Ackerman-Potter analysis was undertaken.

(18) It is evident from Fig 5 that, when enzyme activity is determined at increasing enzyme concentrations in the absence and presence of inhibitor, both curves obtained may be extrapolated to the origin, the intercept of the inhibitor curve is not displaced along the  $X$  axis. Evidence

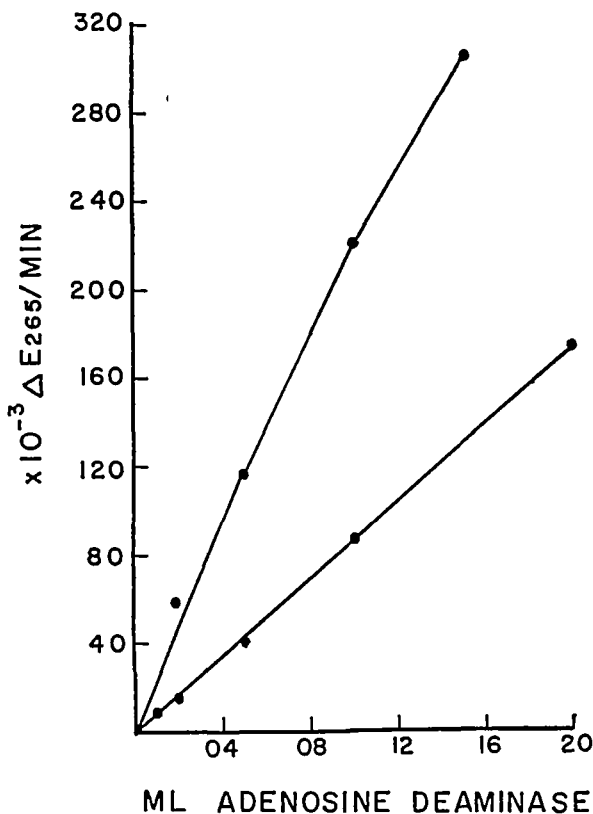


FIG 5 The reaction velocity of adenosine deaminase as a function of enzyme concentration in the absence and presence of 8-azaguanine. To control and experimental cuvettes were added 0.5 ml of  $3.75 \times 10^{-4}$  M adenosine and enough 0.1 M sodium phosphate buffer (pH 7.0) to make the total reaction volume to 2.8 ml. To the experimental cuvettes were added the indicated volumes of a dilute adenosine deaminase preparation, the cuvettes were immediately mixed and the  $E_{265}$  recorded with time. Similar determinations were made in the presence of 0.003 M 8-azaguanine (lower line). ● = the means of duplicate determinations.

therefore indicates that the 8-azaguanine does not titrate the enzyme but that the  $EI$  complex is truly dissociable.

#### DISCUSSION

The inhibition of adenosine deaminase by 8-azaguanine presents several interesting aspects. As seen in Fig 2, the inhibitor concentration curve is sigmoidal. Although a definite interpretation of this shape cannot be made, several possibilities present themselves. The lag in effective inhi-



bition at low 8-azaguanine concentrations might be due to significant guanase-induced destruction of 8-azaguanine at these low inhibitor levels. This seems unlikely since the spectrophotometer tracings show no evidence of an increase in adenosine deaminase activity at increasing time intervals, as one would expect if the inhibitor were being destroyed. A more likely interpretation, inferred from Fig. 4, is that the increasing inhibitory effectiveness per increment inhibitor is due to the combination of increasing numbers of inhibitor molecules per enzyme molecule as the inhibitor concentration rises.

The essentially parallel nature of the lines obtained in the Lineweaver-Burk experiments also suggests that this may not be a typical non-competitive inhibitor. A non-competitive inhibitor which combines with equal affinity with both the free  $E$  and the  $ES$  complex displays an elevation of both intercept and slope on a Lineweaver-Burk reciprocal plot. To the extent to which the inhibitor has lower affinity for free  $E$  than for  $ES$ , the slope is diminished. Thus, true parallel lines should be obtained when the inhibitor is unable to combine with free  $E$  but combines exclusively with  $ES$  complex, yielding the catalytically inactive  $ESI$  complex (17). 8-Azaguanine seems therefore to have a considerably stronger affinity for  $ES$  than for free  $E$ . The effective inhibition caused by a given concentration of 8-azaguanine increased as the adenosine concentration was raised. This increasing inhibition accompanies the increasing conversion of free  $E$  to  $ES$ . Such a phenomenon may have pharmacological significance in that a tissue containing a high steady state adenosine concentration *in vivo* will have more of its adenosine deaminase combined as  $ES$  and may therefore be more susceptible to inhibition by 8-azaguanine than a tissue with a low adenosine concentration.

Tests conducted on various other enzymes to assess the specificity of 8-azaguanine inhibition of adenosine deaminase indicate that erythrocyte nucleoside phosphorylase, yeast acid phosphatase, erythrocyte phosphohexose isomerase, and liver succinoxidase are all completely uninhibited by up to  $10^{-3}$  M 8-azaguanine, whereas xanthine oxidase is strongly inhibited by this compound (19).

The significance *in vivo* of these observations *in vitro* remains to be studied with the use of isotope tracer techniques. The facts that low concentrations of 8-azaguanine give effective inhibition and that the biologically inactive catabolite of 8-azaguanine, 8-azaxanthine, is a considerably weaker inhibitor of adenosine deaminase suggest that this inhibition may be of pharmacological import.

#### SUMMARY

8-Azaguanine was shown to be an inhibitor *in vitro* of adenosine deaminase from rabbit intestinal mucosa and adenocarcinoma 755 with 50

per cent inhibition occurring at  $4 \times 10^{-4}$  M 8-azaguanine, 8-azaxanthine, 6-mercaptopurine, and 2,6-diaminopurine were considerably weaker enzyme inhibitors. The inhibition by 8-azaguanine was found to be non-competitive with regard to substrate, the inhibitor forming a reversible complex with the enzyme. The  $K_i$  and the number of moles of inhibitor which combine per mole of enzyme are not true constants, but vary with the inhibitor concentration. Indirect evidence suggests that the inhibitor manifests higher affinity for the enzyme-substrate complex than for the free enzyme.

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# FLAVOENZYME CATALYSIS SUBSTRATE-COMPETITIVE INHIBITION OF D-AMINO ACID OXIDASE\*

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In an earlier paper from this laboratory (1) there were described the characteristics of competitive inhibition resulting from the interference of certain aromatic compounds with the association of flavin adenine dinucleotide and the separated protein of D-amino acid oxidase. Included among the inhibitors were auramine, quinine, and various other quinolines and anilines. We now present our findings which relate to the structural requirements for some well defined substrate-competitive processes.

The antimetabolic action of benzoate and cinnamate has been demonstrated by their ability to interfere with the oxidation of such metabolites as butyric, crotonic, and acetic acids (2-5) as well as certain L- and D-amino acids (6-8) in kidney and liver slices and homogenates. More recently we reported that benzoate and cinnamate can effect metabolic "shunts" in slices of various organs of the guinea pig and rat. In particular, cinnamate was shown to suppress the oxygen consumption of these tissues and to elicit a marked glycolytic production of lactate. Moreover, the inhibition of aerobic activity was found to be reversible and could be interpreted in terms of a competitive mechanism, embarrassing the oxidation of fatty acids and amino acids (9).

With respect to the action of benzoate on an isolated enzyme system such as D-amino acid oxidase, Klein and Kamin (8) suggested that the inhibition was related to the formation of a benzoic acid-enzyme complex. In more detailed kinetic studies of this antagonism of amino acid oxidation by benzoate, we found that the action of this inhibitor is primarily substrate-competitive (1, 10). This was confirmed by Bartlett in his studies on various substituted benzoates (11). More recently, Klein has reported that certain heterocyclic acids with aromatic properties also can inhibit this system competitively (12).

In the present paper, evidence will be presented that substrate-competitive inhibition of D-amino acid oxidase is not limited to compounds

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with aromatic character, but is associated in general with anions possessing conjugated systems in their structures

### RESULTS AND DISCUSSION

*Competitive Inhibition by Compounds Other Than Benzoate*—Our initial experiments revealed that, in addition to benzoate, such structurally different compounds as crotonate, cinnamate, and indole-2-carboxylate can all compete reversibly with D-alanine for the "complete" enzyme (flavin adenine dinucleotide plus apoenzyme). Appropriate treatment (13) of the kinetic data obtained with these new inhibitors is presented in Figs 1, 2, and 3. In each case it will be seen that, as the ratio of alanine to inhibitor is increased, the degree of inhibition decreases and the same maxi-

TABLE I

*Michaelis Dissociation Constants for D-Alanine and Inhibitor Complexes of D-Amino Acid Oxidase of Lamb and Hog Kidney*

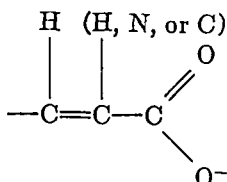
See "Experimental" for analytical details

Inhibitor		$K_s$ mole per l	$K_i$ mole per l
Cinnamate Crotonate	Lamb enzyme	$5.6 \times 10^{-5}$	$7.6 \times 10^{-5}$ $3.8 \times 10^{-5}$
	Hog enzyme	$2.3 \times 10^{-5}$	$2.2 \times 10^{-4}$ $2.1 \times 10^{-5}$ $3.4 \times 10^{-6}$
Cinnamate Benzoate Indole-2-carboxylate			

mal velocity obtains as in the absence of the inhibitor. The relative affinities of the lamb and hog kidney enzymes for D-alanine and these new inhibitors as calculated from the data are given by the Michaelis constants in Table I. These constants show that the enzymes combine with the inhibitors much more avidly than with the substrate, and that there are conspicuous differences among the inhibitors in their degrees of binding. Thus, the weakest inhibitor in the series, cinnamate, is held by the enzyme at least 10 times more tightly than alanine, while the potent indole-2-carboxylate is bound 1000 times more strongly.

This substrate-competitive action of acyclic and heterocyclic compounds constituted the first evidence for the existence of a common inhibitory configuration more general in character than that represented by an aromatic structure such as benzoate. It was cinnamate and, in particular, acrylate and crotonate, the simplest compounds in the series, that provided the clue to the nature of this essential inhibitory structure. The conju-

gate system comprising the crotonate molecule is common to all of the inhibitors described by Figs 1, 2, and 3, suggesting that inhibitory ability derives from the constituent grouping



*Nature of Essential Inhibitory Structure*—In order to probe further into the role of such a structure and its individual parts in the inhibitory mechanism, compounds possessing this constituent grouping, or variations thereof,

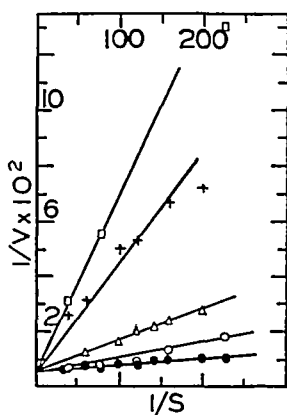


FIG 1

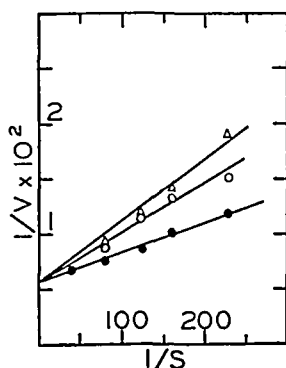


FIG 2

FIG 1 Substrate-competitive inhibition by cinnamate of D-amino acid oxidase of lamb kidney.  $S$  denotes molar concentrations of D-alanine, and  $V$  microliters of  $\text{O}_2$  per 20 minutes. ●, uninhibited process. Molar concentrations of cinnamate: ○,  $5 \times 10^{-5}$ ; Δ,  $1 \times 10^{-4}$ ; +,  $5 \times 10^{-4}$ ; □,  $1 \times 10^{-3}$ .

FIG 2 Substrate-competitive inhibition by crotonate under the same conditions as for Fig 1. ●, uninhibited process. Molar concentrations of crotonate: ○,  $1.33 \times 10^{-4}$ ; Δ,  $2.66 \times 10^{-4}$ .

in a wide variety were examined for activity. The results of this survey are summarized in Tables II and III.

Perhaps the most obvious structural prerequisite for enzyme binding established by the data presented is the carbon-carbon double bond. Thus, unsaturated compounds such as the acrylates, cinnamate, and benzoate are all potent inhibitors, whereas their fully hydrogenated analogues are essentially ineffective. Moreover, this survey lends further support to our conclusions from Figs 1, 2, and 3 that the carbon-carbon double bond can be a part of a variety of structures: aliphatic, aromatic, and heterocyclic.

A second structural requirement for inhibition derived from Tables II

and III is that an anionic group must be located immediately adjacent to the double bond. Whenever such an anionic grouping is eliminated, as in cinnamamide, nicotinamide, hippuric acid, allyl alcohol, etc., the inhibitory activity is markedly diminished or eliminated. The data show further that the anionic groups of the inhibitory compounds may be of markedly different types. Thus, in addition to the carboxylate compounds, phenolates<sup>1</sup> and probably sulfonates also possess activity.

It is significant that the presence of more than one anionic group, carboxylic or hydroxylic, in an unsaturated molecule can eliminate its ability to inhibit. Thus compounds such as fumarate, maleate, and dihydroxyphenols are inactive. This finding is consonant with the established

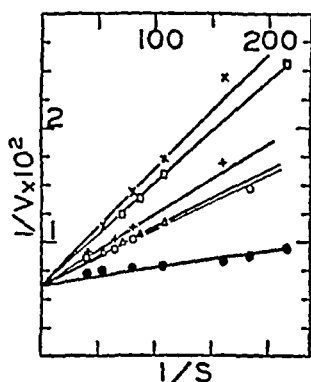


FIG. 3 Substrate-competitive inhibition by indole-2-carboxylate, cinnamate, and benzoate of D-amino acid oxidase of hog kidney. Same conditions as given for Figs. 1 and 2. ●, uninhibited process. Molar concentrations of inhibitors: indole-2-carboxylate, +,  $9.89 \times 10^{-6}$ ; □,  $2.00 \times 10^{-6}$ ; X,  $2.06 \times 10^{-6}$ . Cinnamate, ○,  $5.05 \times 10^{-6}$ ; benzoate, Δ,  $5.15 \times 10^{-6}$ .

fact that the dicarboxylic amino acids, D-glutamate and D-aspartate, have little or no substrate activity in the D-amino acid oxidase system. Although observations such as these are obviously of value in deducing the structure of the substrate-binding surface of the enzyme, their final interpretation depends upon the quantitative differentiation of the intramolecular electronic and steric effects which result from the introduction of a second anionic group into a substrate or inhibitor molecule. The influ-

<sup>1</sup> Krahel *et al.* reported the inhibition of D-amino acid oxidase by trichlorophenol (14). If we subject their data to the Lineweaver-Burk analysis (13), we find that the inhibition is of the reversible substrate-competitive type (calculated  $K_i$ ,  $1.7 \times 10^{-4}$  mole per liter). We have likewise analyzed the earlier data of Klein and Olsen for the inhibition of D-amino acid oxidase by kojic acid (15) and confirm that this inhibition is also substrate-competitive with a calculated  $K_i$  of  $2.2 \times 10^{-6}$  mole per liter (12). It may be pointed out that kojic acid is structurally similar to the phenolic type inhibitors cited in our studies.

TABLE II

*Inhibition of D-Amino Acid Oxidase of Lamb Kidney by  $\alpha,\beta$ -Unsaturated Acid Anions and Substituted Phenols*

DL-Alanine,  $6.25 \times 10^{-3}$  M, inhibitors,  $3.0 \times 10^{-3}$  M Per cent inhibition based on 20 minute  $O_2$  uptakes and calculated as (1 minus inhibited rate divided by uninhibited rate)  $\times 100$  Temperature,  $30^\circ$

Compound	Per cent inhibition	Compound	Per cent inhibition
Aliphatic unsaturated and saturated anions		Phenylsulfonates	
Crotonic acid	99	<i>p</i> -Toluenesulfonic acid	34
Butyric "	0	Metanilic acid	5
Dimethylacrylic acid	70	Sulfanilic "	3
Isovaleric acid	0	Benzoates and naphthoates	
Sorbic acid	93	Benzoic acid	100
Traumatic acid	36	Cyclohexanecarboxylic acid*	53
Lauric acid	0	Hippuric acid	13
Fumaric acid	0	<i>p</i> -Iodosobenzoic acid	87
Maleic "	0	<i>o</i> -Iodosobenzoic "	24
Ascorbic acid	0	<i>p</i> -Methoxybenzoic acid	100
Acetylenedicarboxylic acid	4	<i>o</i> -Methoxybenzoic "	67
Cinnamates and related compounds		3,4,5-Trimethoxybenzoic acid	26
Cinnamic acid	100	<i>p</i> -Carboxybenzoic acid	74
Hydrocinnamic acid*	55	<i>m</i> -Carboxybenzoic "	20
Cinnamamide	0	<i>o</i> -Carboxybenzoic "	3
Mandelic acid*	36	$\beta$ -Naphthoic acid	100
Heterocycles		$\alpha$ -Naphthoic "	14
Nicotinic acid	65	Miscellaneous	
Nicotinamide	6	Phenylacetic acid	15
2-Furoic acid	100	Malonic acid	10
Indole-2-carboxylic acid	100	Acetoacetic acid	0
Phenolates		Alloxan	0
Tribromophenol	40		
Triodophenol	19		
<i>p</i> -Aminophenol	30		
Hydroquinone	73		
$\beta$ -Naphthol	49		
$\alpha$ -Naphthol	19		
Catechol	19		
Resorcinol	0		
2,3-Dihydroxyphenol	0		
3,5-Dihydroxyphenol	5		

\* These inhibitions are probably attributable to a contamination by the unsaturated analogues of these acids. On short standing solutions of purified hydrocinnamic acid and cyclohexanecarboxylic acid are found to decolorize permanganate and bromine solutions.



ence of such factors on the inhibitory mechanism is well illustrated by the variations in inhibitory ability of substituted benzoates in Table II. Within this one family of inhibitors it is apparent that enzyme-inhibitor binding is affected by the size, shape, position, and electronic nature of the substituent group in the phenyl nucleus.

While the data of Tables I and II provide convincing evidence that a variety of anionic unsaturated compounds can be potent competitors with the substrate in the D-amino acid oxidase system, the structural criteria presented by these inhibitors are not considered to be absolute or entirely

TABLE III

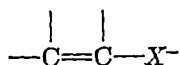
*Inhibition of D-Amino Acid Oxidase of Hog Kidney by  $\alpha,\beta$ -Unsaturated Anions*

DL-Alanine,  $6.25 \times 10^{-3}$  M, inhibitors,  $5.0 \times 10^{-3}$  M Per cent inhibition, calculated as in Table II Temperature,  $37^\circ$

Compound		Per cent inhibition
Acrylates, etc	Acrylic acid	75
	Allyl alcohol	13
	Propionic acid	0
Heterocycles	2,4-Dimethyl-3-carbethoxy-5-carboxypyrrole	22
	N-Methyl-2,4-dimethyl-3-carbethoxy-5-carboxypyrrole	25
	2,4-Dicarbethoxy-3-methyl-5-carboxypyrrole	15
	3-(1,2,4-Trimethyl-5-carbethoxy)pyrrolacrylic acid	7
	Indole-2-carboxylic acid	100
Imino type compounds	$\alpha$ -Oximino $\beta$ -phenylpropionic acid	62
	$\alpha$ -(O-Benzyl)-oximino- $\beta$ -phenylpropionic acid	72
	$\alpha$ -Oximino- $\beta$ -oxo- $\beta$ -(p-chlorophenyl)propionic acid	71
	Hydrazone of pyruvic acid	19
	Oxime of pyruvic acid	18
	Semicarbazone of pyruvic acid	0
"Cinnamate-like" enols	Phenylpyruvic acid	65
	o-Nitrophenylpyruvic acid	32

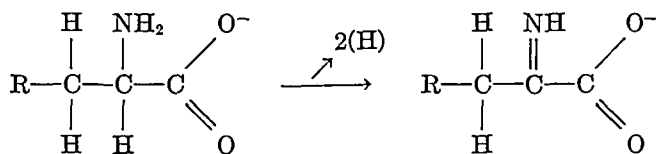
exclusive, other types of compounds may inhibit weakly. The substrate-competitive action of L-leucine, evaluated in this laboratory, can be cited as an example.<sup>2</sup> With a  $K_i$  (13) of  $8.0 \times 10^{-3}$  M, the inhibitory ability in this case is magnitudes weaker than that displayed by compounds in Tables I and II. Similar marginal activities reported for fatty acid anions in high concentrations (16) have been considered insignificant in our work.

The essential inhibitory structure derived from our data may be represented in its most general form as follows



<sup>2</sup> These experiments were performed by Dr. Irving Cooperstein.

where  $X^-$  is a carboxylate or a phenolic hydroxyl group. Excluding an anionic group, the double bond carbons may be substituted with a variety of atoms (C, H, N, etc.) or groups. Although it is clearly demonstrated that such a structure predisposes a compound for reversible combination with the complete D-amino acid-flavoenzyme system in competition with the substrate, it is not immediately apparent why such a mechanism should be operative in amino acid oxidation. Whereas substrate-competitive inhibitors often bear a reasonable resemblance to the substrate in overall structure, there is no such obvious similarity in the present case, and hence much more subtle resemblances must be evaluated. To date, the only attempt to involve finer molecular structures in this inhibitory mechanism had led to the postulate that the benzoate type inhibitors, by virtue of their resonance, possess a charge distribution which is similar to that in the substrate (12). However, the more generalized common inhibitory structure deduced from our present study emphasizes a somewhat more tangible relation between structure and action of the inhibitors in the amino acid oxidation. This relation became apparent when it was recognized that the conjugate system of the inhibitors is strikingly similar to that of the iminoketonic acid product of the oxidation.



The substrate competition would then be considered in a sense to involve this oxidation product. Thus, by combination with the enzyme, the characteristic inhibitor would slow the formation at the enzyme surface of the structurally related imino compound. The kinetic data are not inconsistent with such a postulate.<sup>3</sup>

#### EXPERIMENTAL

D-Amino acid oxidase of lamb kidney was prepared as described by Negelein and Bromel (17) and was stored as a suspension in 40 per cent ammonium sulfate at 0°. For analyses by the usual Warburg technique, a suitable volume of the suspension was centrifuged and the precipitate dissolved in 0.2 M pyrophosphate buffer of pH 8.3. This enzyme solution was added from the side arm to the buffered DL-alanine and inhibitor solutions<sup>4</sup> in the main compartment after a 10 minute period of temperature

<sup>3</sup> This concept originated during a fruitful discussion with Dr. Adrien Albert some years ago.

<sup>4</sup> We have considered the theoretical possibility of enzymatic interaction of ammonia present in small amount in reaction mixtures, and some of the ethylenic compounds employed as inhibitors. We have found no evidence of interference of such a process in the kinetic studies cited here or as yet unpublished.

equilibration (37°) The concentration of pyrophosphate in the final reaction mixture was 0.125 M. Readings were taken every 5 minutes, starting 5 minutes after mixing. Flavin adenine dinucleotide was added in such concentrations so as to achieve a maximal O<sub>2</sub> uptake under the given conditions.

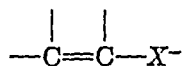
The D-amino acid oxidase of hog kidney was prepared from a powder of acetone-treated cortex by the method of Warburg and Christian (18). The enzyme, precipitated with 13 per cent ammonium sulfate, was dissolved in 0.1 M pyrophosphate at pH 8.3. The resulting solution was lyophilized and then stored over CaCl<sub>2</sub> at 4°. Analyses with this enzyme preparation were carried out under conditions similar to those described for the lamb enzyme.

The pyrrole derivatives were supplied by Dr. A. H. Corwin, and the indole-2-carboxylic acid, phenylpyruvic and o-nitrophenylpyruvic acids, and the propionic acid derivatives by Dr. W. H. Hartung. All other compounds studied were commercial products which were purified when necessary until pertinent physical properties checked with accepted values.

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#### SUMMARY

Kinetic analyses have demonstrated that compounds containing an anionic conjugate system



are predisposed for reversible competition with the substrate in the D-amino acid oxidase system. Acyclic and heterocyclic compounds in a wide variety possessing this constituent grouping, such as acrylates and indole and pyridine derivatives, are shown to equal or surpass the benzoate ion in inhibitory potency. When either the double bond or anionic portion of the inhibitory structure is eliminated, substrate-competitive ability is decreased or eliminated.

The mechanism of the inhibition is discussed in relation to the structural resemblance between the inhibitors and the oxidized amino acid intermediates. It is indicated that the competitions may involve the iminoke-tonic products of oxidation.

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# PLASMA AND URINARY CORTICOSTEROIDS IN THE HYPERTENSIVE FORM OF CONGENITAL ADRENAL HYPERPLASIA\*

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This report describes a systematic study of adrenocortical steroid metabolites in the peripheral blood and urine of an 8 year-old female pseudohermaphrodite with congenital adrenal hyperplasia and systemic hypertension, a form of the disease only recently recognized (1, 2). The findings are reported in detail because they differ from those previously recorded in normotensive subjects with congenital adrenal hyperplasia.  $\Delta^4$ -pregnene-17 $\alpha$ ,21-diol-3,20-dione (Substance S) and pregnane-3 $\alpha$ ,17 $\alpha$ ,21-triol-20-one (tetrahydro S) were detected in the peripheral blood of the subject. Tetrahydro S, pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\xi$ ,21-tetrol, pregnane-3 $\alpha$ ,21-diol-20-one, pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol, pregnane-3 $\alpha$ ,20 $\alpha$ -diol, etiocholan-3 $\alpha$ -ol-17-one, and androstan-3 $\alpha$ -ol-17-one were isolated from the urine. None of the normally present C<sub>21</sub> and C<sub>19</sub> steroids with an oxygen function at C-11 was detected in the blood or urine.

## Methods

**Blood Steroids**—The levels of directly extractable (free) and glucuronidase-liberated (conjugated) plasma corticosteroids were measured by methods previously reported (3, 4).

**Urine Collection and Extraction**—7.5 liters of urine were collected under toluene over a period of 10 days prior to treatment and stored at  $-6^\circ$ . At intervals aliquots were thawed at room temperature and processed in the same manner. The urine was first extracted at pH 7.0 with  $\frac{1}{3}$  volume of methylene chloride on a mechanical shaker. The methylene chloride phase was washed three times with  $\frac{1}{4}$  of its volume of 0.1 N NaOH and washed twice with  $\frac{1}{4}$  of its volume of water and combined to form "Free Extract I." After this extraction, the urine was incubated with  $\beta$ -glucuronidase (Ketodase, 350 units per ml. of urine) in 0.1 M acetate buffer.

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at pH 4.5 for 72 hours and continuously extracted with methylene chloride in a Hershberg-Wolfe type apparatus for 48 hours. The extracts were washed in the same manner and combined to form "Glucuronidase Extract II." Finally, after acid (pH 1.0) hydrolysis at room temperature for 24 hours, the urine was manually extracted three times with  $\frac{1}{2}$  of its volume of methylene chloride and the washed extracts were combined to form "Acid Extract III."

*Preliminary Fractionation of Extracts*—Each of the combined crude extracts was treated with Girard's reagent T at room temperature for 16 hours. The individual ketonic and non-ketonic fractions were again treated with this reagent and the appropriate fractions were combined. The three ketonic fractions so obtained were separately chromatographed on columns of Florisil at a steroid to adsorbent ratio of 1:100 based on weight. The columns were developed with chloroform and increasing concentrations of methanol (from 2 to 24 per cent) in chloroform.

*Chromatography of Neutral 17-Ketosteroids*—The ketonic fractions of the three extracts, which were eluted from Florisil with chloroform and 2 per cent methanol-chloroform mixtures, were combined and treated with digitonin. The non-precipitable,  $\alpha$  fraction was chromatographed on alumina by a minor modification of the method of Lakshmanan and Lieberman (5). The  $\beta$  fraction was assayed for dehydroepiandrosterone by the method of Allen *et al* (6).

*Chromatography of Corticosteroids*—The non-ketonic fractions of the three crude extracts were chromatographed separately on columns of silica gel (7) at a steroid to adsorbent ratio of 1:200. The columns were developed with increasing concentrations of ethanol (from 0.2 to 25.0 per cent) in methylene chloride. Suitable non-ketonic fractions from the silica gel columns and ketonic fractions from the Florisil columns were finally chromatographed on paper. In all cases, 6 by 24 inch strips of Whatman No. 1 filter paper were used, permitting simultaneous chromatography of multiple reference steroids. The solvent systems (8) employed were B<sub>5</sub> (benzene 500 ml, methanol 250 ml, water 250 ml), NB<sub>1</sub> (2,2,4-trimethylpentane 250 ml, toluene 250 ml, methanol 350 ml, water 150 ml), NB<sub>2</sub> (2,2,4-trimethylpentane 165 ml, toluene 335 ml, methanol 300 ml, water 200 ml), E<sub>1</sub> (2,2,4-trimethylpentane 500 ml, methanol 450 ml, water 50 ml), E<sub>2</sub>B (2,2,4-trimethylpentane 500 ml, *tert*-butanol 250 ml, water 450 ml), and E<sub>4</sub> (2,2,4-trimethylpentane 500 ml, *tert*-butanol 225 ml, methanol 225 ml, water 50 ml). Steroids were detected on paper chromatograms by scanning with ultraviolet light and staining with triphenyltetrazolium hydrochloride (TPZ), sodium hydroxide in methanol (9), 4 per cent phosphomolybdic acid in absolute ethanol (w/v), and 15 per cent phosphoric acid (10) solutions.

*Other Methods*—Formaldehyde and acetaldehyde determinations were performed by the method of Cox (11). A modification (4) of the Nelson and Samuels method (12) for the determination of Porter-Silber chromogens (13) was employed. 17-Ketosteroids were assayed by a procedure previously reported (14). The sulfuric acid (15, 16) and 2,4-dinitrophenylhydrazine (17) chromogen spectra were determined with a Beckman DU spectrophotometer. Melting points were obtained on a Fisher-Johns apparatus and are reported uncorrected.

### Results

*Plasma Corticosteroids*—The level of free Porter-Silber chromogens was 24.7  $\gamma$  per 100 ml of plasma and of conjugated, 52.3  $\gamma$  per 100 ml of plasma (normal range in this laboratory, 4.0 to 12.0). The determination was repeated 1 day later and the levels were found to be 26.2 and 32.6  $\gamma$  per 100 ml of plasma. Each extract was chromatographed on paper in the solvent system B<sub>5</sub> of Bush. A single steroid was detected in the free fraction, identical with Substance S in mobility and staining characteristics. A single steroid detectable in the conjugated fraction was identified as tetrahydro S by the same criteria.

### Ketonic C<sub>21</sub> Steroids in Urine

*Isolation of Tetrahydro S*—The ketonic fraction of "Glucuronidase Extract II" weighed 728 mg and contained 172 mg of Porter-Silber chromogenic and 193 mg of formaldehydogenic material. 165 mg of material measured by the Porter-Silber reaction were eluted from Florisil with 10 per cent methanol in chloroform. This amorphous material, containing considerable pigment, was chromatographed on thirty sheets of paper in system B<sub>5</sub>. A single steroid of the polarity and with the staining characteristics of tetrahydro S appeared to be present. The eluted substance was similar to tetrahydro S in chromatographic behavior in solvent systems NB<sub>2</sub>, E<sub>2</sub>B, and E<sub>4</sub>. One-fifth of the eluted steroid was chromatographed on a column of silica gel. Crystalline material was eluted with 4.5 per cent ethanol in methylene chloride. Several recrystallizations from methanol yielded 26 mg of steroid with a constant melting point of 184–185°. The melting point was not depressed by admixture of authentic tetrahydro S. 1.2 mg of the steroid and 0.8 mg of authentic tetrahydro S were separately acetylated with acetic anhydride in pyridine at room temperature for 16 hours. Chromatographed on paper in system E<sub>1</sub>, both were shown to consist of an identical mixture of a monoacetate and diacetate. Acetylation was repeated, crystallization from ethanol yielded in each instance a product melting at 200–204°, mixture m.p. 200–204°, reported melting point of 3 $\alpha$ ,21-diacetoxypregnan-17 $\alpha$ -ol-20-one 201–206° (18).



The sulfuric acid chromogen spectrum of both the free alcohol and the diacetate consisted of a peak between 310 and 315  $m\mu$  and a plateau extending from 400 to 430  $m\mu$ . Infrared spectroscopy of the acetylated compound in carbon disulfide revealed carbonyl-stretching bands at 1753, 1736, 1240, and 1230  $\text{cm}^{-1}$ . The fingerprint bands were identical to those of  $3\alpha,21$ -diacetoxypregnan- $17\alpha$ -ol-20-one. Identity of the compound was further established by oxidation with chromium trioxide in glacial acetic acid. Paper chromatography of the oxidized product in solvent system  $E_1$  demonstrated a substance with the mobility, staining characteristics, and sulfuric acid chromogen spectrum of etiocholan-3,17-dione.

Paper chromatography of the ketonic fraction of Free Extract I (weight 12.1 mg, Porter-Silber chromogens 1.0 mg, formaldehydogenic compounds 3.0 mg) led to the recovery of 0.5 mg of tetrahydro S. Chromatography of the ketonic fraction of Acid Extract III (weight 15.4 mg, Porter-Silber chromogens 17.6 mg, formaldehydogenic compounds 30.0 mg) yielded an additional 15 mg of the same steroid. The ketonic fractions of the three crude extracts, accordingly, contained approximately 180 mg of tetrahydro S. By calculation, the excretion of this steroid in the urine averaged 24 mg per liter or 18 mg per 24 hours.

*Isolation of Tetrahydrodesoxycorticosterone*—On the multiple paper chromatograms of the 2 per cent ethanol fractions obtained from Florisil chromatography of Glucuronidase Extract II, material stained by TPZ and less polar than tetrahydro S was also detected in small amount. The appropriate zones were eluted with methanol, combined, and chromatographed on four sheets of paper in solvent system  $NB_2$ . In this system the material was completely separated from traces of tetrahydro S. Like tetrahydro S it did not absorb ultraviolet light or react with sodium hydroxide in methanol, however, it stained intensely with TPZ after heating at  $90^\circ$  (19). On heating with the phosphoric acid reagent, a pink fluorescence was visible in ultraviolet light. 4.5 mg of formaldehydogenic material along with considerable pigment were eluted from paper with methanol. It was chromatographed on paper in solvent system  $E_4$ , in which system it migrated more rapidly than desoxycorticosterone. Again eluted from paper it was chromatographed on a column of 4 gm of silica gel. The column was developed with increasing concentrations (from 0.2 to 10.0 per cent) of ethanol in methylene chloride. Crystalline material was eluted at ethanol concentrations between 1.5 and 2.0 per cent. After recrystallization from mixtures of methanol-methylene chloride, 3.2 mg of fine crystals were recovered which melted at  $150$ – $153^\circ$ . A sample of authentic pregnane- $3\alpha,21$ -diol-20-one melted at  $152$ – $153^\circ$ , mixture m.p.  $150$ – $152^\circ$ . The two were identical by paper chromatography in solvent systems  $NB_2$  and  $E_4$ . The sulfuric acid chromogen spectrum consisted of

a peak at 320  $m\mu$  and a plateau extending from 380 to 400  $m\mu$ . The steroid reacted with 2,4-dinitrophenylhydrazine to yield an orange product with maximal absorption at 495  $m\mu$ . 0.5 mg of the steroid was acetylated with acetic anhydride in pyridine at room temperature for 16 hours and subsequently chromatographed on paper in system  $E_1$ . A zone was detected at 36.3 to 38.2 cm from the origin, compared to 21-acetoxypregnan-17 $\alpha$ -ol-3,20-dione at 2.5 to 5.6 cm and 3 $\alpha$ ,21-diacetoxypregnan-17 $\alpha$ -ol-20-one at 18.3 to 21.8 cm, simultaneously chromatographed. The mobility of the acetate was identical with that of 3 $\alpha$ ,21-diacetoxypregnan-20-one.

By infrared spectrometry the acetylated steroid was identical with authentic 3 $\alpha$ ,21-diacetoxypregnan-20-one. Identity was further established by oxidation with chromium trioxide. 0.5 mg of steroid was treated with 1.0 mg of chromium trioxide in 2.0 ml of glacial acetic acid at 45° for 1 hour. The mixture was adjusted to pH 11.0 with 4.0 N NaOH and extracted with methylene chloride. The aqueous phase was adjusted to pH 1.0 with 40 per cent  $H_2SO_4$ , and the mixture was extracted with ether. The ether phase was washed with water, dried over anhydrous  $Na_2SO_4$ , and evaporated. Several recrystallizations of the yellowish residue from mixtures of acetone, ethanol, and 2,2,4-trimethylpentane led to the isolation of 0.2 mg of white crystals melting at 142–145°. The melting point was not depressed by admixture of authentic 3-ketoetianic acid, m.p. 144–147°.

*Partial Characterization of Other Ketonic  $C_{21}$  Steroids*—Trace amounts of material were eluted in the later fractions during Florisil chromatography of ketonic extracts I to III. These were combined, successively chromatographed on paper in solvent systems  $B_5$  and  $E_2B$ , and finally, after acetylation, in systems  $NB_1$  and  $E_1$ . Several apparently pure steroids were isolated and partially characterized by melting point, sulfuric acid chromogen spectrum, and degradation studies. Because of the small quantity and lack of reference standards, none could be completely identified, but all appeared to lack an oxygen function at C-11 by the phosphoric acid-staining method. No traces of the normal urinary metabolites, pregnane-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,21-tetrol-20-one and pregnane-3 $\alpha$ ,17 $\alpha$ ,21-triol-11,20-dione, were found.

#### *Non-Ketonic $C_{21}$ Steroids in Urine*

*Isolation of Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\xi$ ,21-tetrol*—69.2 mg of semicrystalline material were eluted with 6 per cent ethanol in methylene chloride during silica gel chromatography of the non-ketonic fraction of Glucuronidase Extract II. This fraction contained 45.0 mg of formaldehydogenic material and failed to react with either blue tetrazolium or the Porter-Silber reagent. Attempts at crystallization were unsuccessful. 18.0 mg of

steroid, as measured by the periodate reagent, were chromatographed on four sheets of paper in system B<sub>5</sub>. No material was detected with ultraviolet light, TPZ, or sodium hydroxide in methanol. The phosphomolybdic acid reagent stained a zone 12.5 to 15.0 cm from the origin, compared to hydrocortisone at 9.4 to 12.6 cm and tetrahydro S at 22.5 to 25.0 cm. The zone reacted with phosphoric acid and heat to produce a violet color with pink fluorescence in ultraviolet light. The same reagent stained faintly a second zone 25.0 to 28.9 cm from the starting line. Material in the less polar zone was found not to be formaldehydogenic and was set aside for future study. The more polar zone was eluted with mixtures of methanol and ethyl acetate. It was chromatographed on a column of 10 gm of silica gel and eluted in a semicrystalline state with 7.5 per cent ethanol in methylene chloride. These fractions were combined, leached with acetone, and recrystallized from ethanol to yield 14.5 mg of colorless crystals which softened slightly at 239°, solidified at 245°, and melted at 254–258°.

C<sub>21</sub>H<sub>36</sub>O<sub>4</sub>. Calculated, C 71.70, H 10.24, found, C 71.10, H 10.20

A solution of the steroid in concentrated sulfuric acid produced an immediate pink color which gradually turned orange, the absorption spectrum consisted of a small peak at 350 mμ and two major, equal peaks at 445 mμ and 515 mμ. Addition of tap water to the acid solution produced an evanescent bright rose color, as has been noticed to occur with other steroids possessing vicinal hydroxyl groups at C-17 and C-20. 4.5 mg of steroid were acetylated with acetic anhydride in pyridine and chromatographed on paper in system E<sub>1</sub>. Two zones were detected with phosphoric acid, one (16.2 to 19.2 cm from the starting line) at the position of 3α,21-diacetoxypregnan-17α-ol-20-one (16.0 to 18.6 cm) and the second nearer the solvent front (32.7 to 36.4 cm). Both possessed an identical sulfuric acid chromogen spectrum (peak at 310 mμ and lesser plateaus extending from 380 to 400 mμ and from 430 to 440 mμ). The more polar zone was eluted. Repeated acetylation and chromatography demonstrated that this substance was an incompletely acetylated form of the less polar acetate. The polyacetate was eluted from paper and chromatographed on 2.0 gm of silica gel, from which it was eluted with 0.4 per cent ethanol in methylene chloride. It was recrystallized from acetone-2,2,4-trimethylpentane mixtures. The small white crystals became transparent at 75–80° and melted at 147–151°. Infrared spectroscopy of the acetylated compound in carbon disulfide and chloroform solution showed the presence of a free hydroxyl group and the C=O and C—O bands characteristic of acetate groups. The absorption bands in the fingerprint region differed from those of any steroid in the collection at the Sloan-Kettering Institute for Cancer

**Research** The spectrum was consistent with either of the isomers of  $3\alpha$ -,  $20\beta$ -,  $21$ -triacetoxypregnan- $17\alpha$ -ol

Since no reference standard was available, it was necessary to degrade the compound to a known  $17$ -ketosteroid. Periodate oxidation yielded a  $17$ -ketosteroid identical with etiocholan- $3\alpha$ -ol- $17$ -one in chromatographic properties and sulfuric acid chromogen spectrum. The compound was also oxidized with chromium trioxide in glacial acetic acid and the product was chromatographed on paper in solvent system  $E_1$ . Tetrahydro  $S$  and etiocholan- $3\alpha$ -ol- $17$ -one were carried through the same procedure. A single steroid of the polarity and having the sulfuric acid chromogen spectrum of etiocholane- $3$ ,  $17$ -dione was found in each instance. Configuration of the hydroxyl group at  $C-20$  has not yet been established.

*Isolation of Pregnane- $3\alpha$ ,  $20\alpha$ -diol*—Pregnane- $3\alpha$ ,  $20\alpha$ -diol was isolated from the 2 per cent ethanol fractions during silica gel chromatography of the non-ketonic fraction of Glucuronidase Extract II. After leaching with acetone and recrystallization, 14.0 mg of steroid were recovered. Acetylation yielded a substance identical to pregnane- $3\alpha$ ,  $20\alpha$ -diol diacetate by infrared spectroscopy.

*Isolation of Pregnane- $3\alpha$ ,  $17\alpha$ ,  $20\alpha$ -triol*—This compound, present in the non-ketonic fraction of Glucuronidase Extract II, was eluted from the silica gel column with 5 per cent ethanol in methylene chloride. Acetaldehyde was liberated by periodate oxidation. Identification was based upon the chromatographic properties and sulfuric acid chromogen spectrum of the steroid. The subject excreted an average of 6.6 mg of pregnane- $3\alpha$ -,  $17\alpha$ ,  $20\alpha$ -triol per 24 hours, as measured by a method previously reported (20).

*Isolation of Urinary 17-Ketosteroids*—The ketonic fractions of the three crude extracts containing  $17$ -ketosteroids, eluted from the Florisil columns with chloroform and 2 per cent methanol in chloroform, were combined and weighed 370 mg. By assay with the Zimmermann reagent this extract contained 225 mg of  $17$ -ketosteroids. 55 mg of Zimmermann-reacting material were treated with digitonin, the precipitate was recovered, hydrolyzed with pyridine, and assayed colorimetrically. By the Allen method the crude extracts were found to contain a total of 6.2 mg of dehydroepiandrosterone. One-half of the non-precipitable,  $\alpha$  fraction was chromatographed on alumina. Only two  $17$ -ketosteroids were recovered and identified by infrared spectroscopy, androstan- $3\alpha$ -ol- $17$ -one (4.2 mg) and etiocholan- $3\alpha$ -ol- $17$ -one (15.2 mg). No material was eluted in those fractions normally containing  $17$ -ketosteroids oxygenated at  $C-11$ . By calculation, 152.0 mg of etiocholan- $3\alpha$ -ol- $17$ -one and 43.0 mg of androstan- $3\alpha$ -ol- $17$ -one were present in the combined extracts.

*Steroid Excretion during Hydrocortisone Administration*—Urine was col-

lected from the same subject 8 weeks after initiation of treatment with hydrocortisone (40 mg per day) at a time when the blood pressure was normal. 500 ml of urine were incubated with  $\beta$ -glucuronidase, extracted with methylene chloride, and chromatographed on paper in solvent system B<sub>5</sub>. The major zones were detected with TPZ and consisted of tetrahydro E and tetrahydro F, identified by position, staining characteristics, and sulfuric acid chromogen spectra. Two smaller zones were identified as pregnane-11 $\beta$ ,17 $\alpha$ ,21-triol-3,20-dione and pregnane-17 $\alpha$ ,21-diol-3,11,20-trione by the same criteria.

#### DISCUSSION

The major C<sub>21</sub> steroids present in the urine of a subject with congenital adrenal hyperplasia and hypertension were tetrahydro S, pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\xi$ ,21-tetrol, pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol, and pregnane-3 $\alpha$ ,20 $\alpha$ -diol. Reichstein's Substance S and tetrahydro S were detected in the peripheral blood. Tetrahydro S has previously been isolated from urine in cases of adrenal tumor (18, 21) and after the administration of Substance S (22). Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\xi$ ,21-tetrol has not previously been described. By analogy with the recently discovered transformation products of hydrocortisone and cortisone (23), it is considered to be a metabolite of Substance S. Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol has been shown to be a metabolite of 17-hydroxyprogesterone (24, 25) but has also been isolated from urine following the administration of pregnane-17 $\alpha$ ,21-diol-3,20-dione (26). Accordingly, since the latter is a metabolite of Substance S, some of the pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol found in this urine may also be derived from Substance S. Pregnane-3 $\alpha$ ,21-diol-20-one has been found in human urine only after the administration of desoxycorticosterone (22). Desoxycorticosterone might be the precursor of the pregnane-3 $\alpha$ ,20 $\alpha$ -diol excreted by this surgically castrated subject, since this conversion has been demonstrated *in vivo* (27). Origin of the major 17-ketosteroid isolated from the urine, etiocholan-3 $\alpha$ -ol-17-one, is uncertain. Excretion of this compound has been reported in some instances to rise after the administration of Substance S (28). It may be similarly derived in this subject.

The findings of the present study differ strikingly from what has been described in normotensive subjects with congenital adrenal hyperplasia. In the latter, the Porter-Silber chromogen levels of plasma are usually low (25, 29) in spite of increased endogenous adrenocorticotropin production (30), the metabolites of hydrocortisone are found in the urine in only small amount (31). There is characteristically a greatly increased excretion of pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol (31) and of 11-oxygenated 17-keto steroids (31). Such findings have strongly suggested (25, 31) a block in the synthesis of hydrocortisone by the adrenal cortex between 17-hydroxy-

progesterone and Substance S, according to the scheme of Hechter and Pincus (32), due to deficiency of adenal "21-hydroxylase"

In marked contrast, this subject, although also unable to synthesize hydrocortisone as proved by the absence of hydrocortisone or its metabolites in the blood and urine, was able to synthesize Substance S in large amount. The metabolites of Substance S, rather than pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol were predominant in the urine. No 11-oxygenated C<sub>19</sub> or C<sub>21</sub> steroids were detected. Accordingly, in this subject there would appear to be a specific and essentially complete deficiency of the adenal enzyme or enzyme complex concerned with hydroxylation of the steroid molecule at C-11 rather than at C-21. There is reason to believe that the adrenocortical enzyme "11 $\beta$ -hydroxylase," first described by Hayano, Dorfman, and Yamada (33) may be characteristically deficient in the syndrome of congenital adrenal hyperplasia complicated by hypertension. Chromatography of the 17-ketosteroids excreted by a similar subject revealed, precisely as in the present study, a preponderance of etiocholan-3 $\alpha$ -ol-17-one and the absence of any 11-oxygenated steroids<sup>1</sup>

#### SUMMARY

Steroids with the properties on paper chromatograms of Reichstein's Substance S and of tetrahydro S were detected in the peripheral blood of a subject with congenital adrenal hyperplasia and hypertension. Tetrahydro S, pregnane-3 $\alpha$ ,21-diol-20-one, pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\xi$ ,21-tetrol, pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol, and pregnane-3 $\alpha$ ,20 $\alpha$ -diol were isolated from the urine and characterized. The predominant 17-ketosteroid in the urine was etiocholan-3 $\alpha$ -ol-17-one. No 11-oxygenated C<sub>21</sub> or C<sub>19</sub> steroids were detected in either the blood or urine. The abnormal steroid metabolites disappeared from the urine during administration of hydrocortisone. These findings suggest an essentially complete deficiency of adenal "11 $\beta$ -hydroxylase" in the hypertensive form of congenital adrenal hyperplasia.

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# THE METABOLISM OF HYDROXYPROLINE- $\alpha$ -C<sup>14</sup> IN THE INTACT RAT RADIOACTIVITY IN AMINO ACIDS FROM PROTEINS\*

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Since hydroxyproline constitutes 13 per cent of collagen, and collagen-like extracellular protein has been estimated to form about 40 per cent of total body protein (1), hydroxyproline, being therefore over 5 per cent of body protein, represents an important dietary constituent in carnivores. That its metabolism has not received much attention may be due to its unique property of being located almost exclusively in collagen, a protein having practically no turnover (1). Hence, it is considered to be a structural rather than a functional amino acid that, once laid down in protein, will not reappear again in the organism.

Hydroxyproline has long been known to be glycogenic (2). Stetten (3) has shown that hydroxyproline is extensively metabolized in a manner different from that of proline. Womack and Rose (4) demonstrated that it was not converted to arginine, although proline was to some extent. Gianetto and Bouthillier (5), with C<sup>14</sup>-labeled hydroxyproline, found labeled glutamic and aspartic acids as products of hydroxyproline metabolism.

In the present study, alanine was shown to be the principal labeled amino acid, and is derived from hydroxyproline in a fairly direct manner. It was concluded that aspartic and glutamic acids are the products of a second catabolic pathway, with aspartic acid situated closer to hydroxyproline than glutamic acid.

## EXPERIMENTAL

*Measurements of Radioactivity*—Whenever pure, crystalline compounds in sufficient quantity were obtained (Tables III and V), radioactivity was determined by combustion to carbon dioxide in a Pregl microcombustion apparatus. The amount of gas was measured manometrically and the

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radioactivity assayed by a vibrating reed electrometer, as previously described (6) Rat tissue and urine (Table II) were converted to carbon dioxide by a wet combustion method with the Van Slyke-Folch mixture (7) For fractions eluted from column or paper chromatograms, an internal gas flow counter was used, with samples spread on planchets A conversion factor from one instrument to the other was obtained by the use of a planchet containing a standard amount of radioactivity

*DL-Hydroxyproline- $\alpha$ -C<sup>14</sup>*—Diethyl malonate-2-C<sup>14</sup>, obtained from the Nuclear Instrument and Chemical Corporation (3 mmoles), diluted to 6 mmoles with non-radioactive diethyl malonate and dissolved in diethyl carbonate (30 ml), was added to dry sodium ethoxide (6 mmoles) The mixture was stirred for 10 minutes, evaporated nearly to dryness under reduced pressure, and dissolved in 20 ml of diethyl carbonate The resulting sodiodiethyl malonate was made to react with 3-phthalimido-1,2-epoxypropane by the method of Gaudry and Godin (8), diethyl carbonate being substituted for ethanol as the solvent

The 2-carbethoxy-5-phthalimido-4-valerolactone produced was chlorinated without prior isolation by heating to 70° with 0.9 ml of sulfuryl chloride in 15 ml of glacial acetic acid for 2 hours The acetic acid and excess sulfuryl chloride were removed under reduced pressure, and the oily residue was hydrolyzed by refluxing with 20 ml of glacial acetic acid and 20 ml of concentrated hydrochloric acid for 3 hours The two acids were removed under reduced pressure, the residue was dissolved in water, and phthalic acid filtered off The filtrate was neutralized with barium hydroxide (1.2 gm), and an excess of barium hydroxide (1.2 gm) was added This mixture was refluxed 6 hours and filtered cold, the filtrate being neutralized with hydrochloric acid and passed through a short column of ion exchange resin Amberlite IR-120 in the acid form Hydroxyproline was eluted from the column with 200 ml of 2 N ammonia and the eluate taken to dryness under reduced pressure The residue was dissolved in water and refluxed for 1 hour with an excess of copper carbonate This mixture was filtered hot and the volume reduced to 1 ml *in vacuo* The copper salt of *DL*-hydroxyproline crystallized and was filtered and washed twice with 1 ml portions of cold water The crystals were dissolved in dilute acetic acid and the copper salt was decomposed with hydrogen sulfide The copper sulfide was removed by filtration, the filtrate evaporated to 1 ml under reduced pressure, and ethanol added until precipitation just began Upon cooling, *DL*-hydroxyproline- $\alpha$ -C<sup>14</sup> crystallized and was recrystallized in a similar manner, m p 238–250°, specific activity 664  $\mu$ c per millimole, yield 134.3 mg or 17.1 per cent Specific activity of the copper salt was 668  $\mu$ c per millimole, while an isotope dilution assay of the *DL*-hydroxyproline gave a specific activity of 655  $\mu$ c per millimole Paper chromatog-

raphy and radioautography of the copper salt showed the presence of a small amount of DL-allohydroxyproline- $\alpha$ -C<sup>14</sup> (4.8 per cent)

*DL-Allohydroxyproline- $\alpha$ -C<sup>14</sup>*—This compound was isolated as the copper salt after crystals of the DL-hydroxyproline copper salt were removed (see above). The copper salt of the allo isomer was treated in the same way as that of the natural isomer and yielded the free allohydroxyproline- $\alpha$ -C<sup>14</sup> which was isolated in a similar manner to the natural compound.

*Isolation Methods*—Rat I and Rat II (both male albino, 190 gm) were injected intraperitoneally with DL-hydroxyproline- $\alpha$ -C<sup>14</sup> (15.0 mg) and DL-allohydroxyproline- $\alpha$ -C<sup>14</sup> (15.1 mg), dissolved in 2 ml of saline, respectively, and placed in separate glass metabolism cages. Expired carbon dioxide was collected in alkali at regular intervals, precipitated as barium carbonate, and assayed for radioactivity. Urine was also collected and assayed. After 4 hours and 20 minutes (Rat I) and 3 hours and 50 minutes (Rat II), the two rats were killed by decapitation and bled. The livers (6.4 gm, Rat I, 7.0 gm, Rat II) were cleaned, homogenized with 15 ml of a 10 per cent trichloroacetic acid, then suspended in acetone, and finally in ether, yielding dry protein (1.425 gm, Rat I, 1.496 gm, Rat II). The remainder of the two carcasses was saved and radioactivity of samples of the muscle and tendon protein from both rats was measured. Radioactivity of liver protein, the trichloroacetic acid filtrates ("non-protein fraction"), and the acetone and ether extracts ("fat-soluble fraction") was measured. Glycogen was isolated by alcohol precipitation from the first trichloroacetic acid filtrate, and assayed.

The liver protein of each of the two rats was divided into two fractions, and each fraction was hydrolyzed for 24 hours with 6 N hydrochloric acid (35 ml). The first fraction (A) from each rat was hydrolyzed in the presence of carrier quantities of glutamic acid, aspartic acid, proline, hydroxyproline, and glycine. Fraction B from each rat was hydrolyzed without addition of carrier amino acids. After hydrolysis, the excess of hydrochloric acid was removed from all four fractions under reduced pressure, and each fraction was decolorized with charcoal. The two B fractions were made up to 10 ml with 60 per cent ethanol and used in chromatography and radioautography. Ion exchange resin Amberlite IRA-400 in the hydroxyl form was added to the A fractions to pH 7.0. Glutamic and aspartic acids were absorbed by the resin, while the other amino acids remained in the filtrate (filtrate 1). The two acidic amino acids were eluted with dilute hydrochloric acid. Glutamic acid hydrochloride was crystallized from a concentrated hydrochloric acid solution. Aspartic acid was precipitated as the copper salt. Radioactivity of the free acids as well as their salts was measured. Phenylhydantoin derivatives were prepared as previously described (6).

Filtrate 1 was passed through a column of Amberlite IRC-50 resin at pH 4.4 to remove the basic amino acids. The eluate from this column (eluate 2), containing the neutral amino acids, was concentrated to dryness under reduced pressure, and made up to 10 ml with hydrochloric acid (0.25 N), and proline was precipitated as the rhodanilate by the method of Bergmann (9). The salt was isolated and assayed for radioactivity. The phenylhydantoin derivative was prepared and assayed. After the removal of the proline rhodanilate, the hydroxyproline reineckate was prepared and isolated (9). The salt was decomposed with pyridine and hydroxyproline recrystallized from water with the gradual addition of alcohol and assayed.

Alanine, glycine, and serine were isolated by chromatographing the B fraction (protein hydrolysate without carrier) on a Dowex 50-X8 (200-400 mesh) resin column, identical with that used in experiments previously described (10). Elution was effected by 2 N hydrochloric acid. Radioactive fractions were further purified by chromatography on large sheets of filter paper and elution of the bands of the respective compounds. No appreciable amounts of radioactivity were found in any amino acids other than those shown in Tables III and V. For assay, degradation, and preparation of derivatives, the eluted radioactive compounds were mixed with non-radioactive carrier.

Urine and the trichloroacetic acid-soluble, non-protein liver fraction were each subjected to two-dimensional paper chromatography. Radioautography revealed the principal radioactive metabolites.

*Degradation Experiments*—Glutamic and aspartic acids, after dilution with carrier, were degraded as previously described (6).

Alanine (16.3 mg) was degraded in an aqueous solution containing 200 mg of citrate buffer, pH 2.5, and 200 mg of ninhydrin. The mixture was boiled for 5 minutes and the evolved gases were swept with helium into two traps cooled with liquid nitrogen. Sweeping was continued for 15 minutes. The traps, being part of a vacuum line, were then evacuated to  $10^{-4}$  mm mercury and their contents distilled at room temperature into a vessel containing dimethylphenylhydrazine (37 mg) in glacial acetic acid (2 ml), cooled with liquid nitrogen, and also evacuated. When the distillation was complete, the vessel was closed off and warmed to room temperature, and the contents were stirred with a magnetic stirrer for 1 hour. The vessel was then again cooled with liquid nitrogen and the carbon dioxide distilled into an evacuated gas bulb cooled with liquid nitrogen by raising the temperature surrounding the vessel to  $-100^{\circ}$ . The amount of carbon dioxide in the gas bulb was measured manometrically and its radioactivity determined on the electrometer. The vessel containing the dimethylphenylhydrazone of acetaldehyde was warmed to room temperature, detached from the vacuum line, and the precipitate removed by filtration.

It was recrystallized twice from ethanol and assayed for carbon and for radioactivity

Glycine was degraded on the vacuum line in a manner identical to that used for alanine, except that only the carbon dioxide was swept out of the reaction vessel. The formaldehyde remaining was transformed into the dimedon derivative and purified by crystallization from ethanol for assay

## RESULTS AND DISCUSSION

*Radioactivity in Expired Air, Liver, and Urine*—The activity in expired carbon dioxide was surprisingly high (Table I), almost twice the value ob-

TABLE I  
*Radioactivity in Expired Carbon Dioxide*

The dose injected was 73.4  $\mu\text{c}$  of DL-hydroxyproline (Rat I) and 73.8  $\mu\text{c}$  of DL-allohydroxyproline (Rat II), specific activity, 664  $\mu\text{c}$  per millimole

Times of collection	Rat I					Rat II				
	Activity	Per cent of dose	Specific activity	Activity, cumulative	Per cent of dose, cumulative	Activity	Per cent of dose	Specific activity	Activity, cumulative	Per cent of dose, cumulative
hrs	$\mu\text{c}$		$\mu\text{c per mmole}$	$\mu\text{c}$		$\mu\text{c}$		$\mu\text{c per mmole}$	$\mu\text{c}$	
1	5.544	7.6	0.413	5.544	7.6	2.442	3.3	0.214	2.442	3.3
2	5.916	8.1	0.510	11.460	15.6	3.024	4.1	0.304	5.466	7.4
3	3.438	4.7	0.336	14.898	20.3	2.064	2.8	0.214	7.530	10.2
3 $\frac{5}{8}$						1.178	1.6	0.143	8.708	11.8
4*	2.638	3.6	0.253	17.536	23.9	0.238	0.3	0.143	8.946	12.1
4 $\frac{1}{2}$	0.878	1.2	0.253	18.414	25.1					

\* Calculated value

tained with DL-histidine- $\alpha\text{-C}^{14}$  in 4 hours (6), and showed only a gradual leveling off towards the end of that period. This effect may be due to the dual metabolic pathway of hydroxyproline through alanine and through aspartic and glutamic acids, as postulated below. The animal which received allohydroxyproline appeared to expire only about half the activity in carbon dioxide in the same time (Table I), a fact in accord with the absence of the alanine pathway for allohydroxyproline.

The data in Table II reveal the incorporation of radioactivity into liver fractions and urine. The incorporation of activity into glycogen is comparatively high, reflecting, no doubt, the pathway to alanine which, by transamination to pyruvate, would readily connect to glycogen. It is significant that here, again, the glycogen value for the animal given the allo form is about one-half that for the animal receiving the natural isomer.

The relatively high activity in the liver non-protein fraction is due partly to hydroxyproline and partly to one major metabolite, so far unidentified, as revealed by paper chromatography ( $R_F$  in phenol-water, 0.32, in lutidine-water, 0.58). It differs from  $\gamma$ -hydroxyglutamic acid (11) and from  $\gamma$ -hydroxyornithine (12), nor did it show reactions characteristic for  $\gamma$ -hydroxyglutamic semialdehyde or hydroxypyrrolinecarboxylic acid.

The major portion of activity (about 30 per cent) in the urine, apart from hydroxyproline, was centered in one metabolite ( $R_F$  in phenol-water, 0.73, in lutidine-water, 0.66), different from the liver metabolite mentioned above and differing also from  $\gamma$ -hydroxyglutamic acid,  $\gamma$ -hydroxyornithine, and acetoacetic acid. It was found to be unstable in acid solution and slightly volatile. Preliminary observations showed it to be

TABLE II  
Gross Metabolism

The dose injected was 73.4  $\mu$ c of DL-hydroxyproline (Rat I) and 73.8  $\mu$ c of DL-allohydroxyproline (Rat II), specific activity, 664  $\mu$ c per millimole

Fraction	Rat I		Rat II	
	Activity	Per cent of dose	Activity	Per cent of dose
	$\mu$ c		$\mu$ c	
Liver protein	0.523	0.71	0.308	0.42
“ non-protein	1.455	1.99	1.1015	1.59
“ fat-soluble	0.109	0.15	0.063	0.09
“ glycogen	0.220	0.30	0.120	0.16
Urine	13.970	19.10	22.470	30.50

identical with pyrrole- $\alpha$ -carboxylic acid, already known as the product of D-amino acid oxidase and catalase action on D-hydroxyproline (13). Details of these and further experiments with the urinary metabolites will be the subject of a future publication.

It is noteworthy that the urine of the animal which received the allo form had more than twice the activity of the animal receiving the natural isomer, in view of the fact that all other fractions examined (carbon dioxide, glycogen, protein, etc.) had much lower activities.

Hydroxyproline excreted in urine was isolated by chromatography on the Dowex 50-X8 column described in the experimental section above. It was assayed for the amount of hydroxyproline present (0.06 mg per ml of urine) by the method of Troll and Cannan (14), and for radioactivity, and showed a specific activity (471  $\mu$ c per millimole) not much lower than that of the hydroxyproline injected (664  $\mu$ c per millimole). This finding is in support of the conclusion of Stetten (3) that hydroxyproline *in vivo* is derived from proline by hydroxylation in a peptide-bound form, so that

no free hydroxyproline would exist to dilute the radioactive compound. Very little hydroxyproline would be derived from catabolic reactions of protein, since collagen, the only protein known to contain hydroxyproline, has practically no turnover (1). Experiments are under way to determine more directly whether or not there exists a free hydroxyproline pool *in vivo*.

*Amino Acids from Protein*—The highest activity among the amino acids resided in alanine (Table III). Degradation showed (Table IV) that it

TABLE III  
*Amino Acids from Liver Protein*

The dose injected was 73.4  $\mu$ c of DL-hydroxyproline, specific activity, 664  $\mu$ c per millimole, liver protein dry weight, 1.425 gm, duration of experiment, 4 hours and 20 minutes.

Amino acids and derivatives	Amount present*	Amount of carrier added	Specific activity of diluted compound	Calculated specific activity of undiluted amino acid	Total activity of amino acid in protein	Per cent of dose
	mg	mg	$\mu$ c per mmole $\times 10^{-2}$	$\mu$ c per mmole	$\mu$ c	
Alanine	85.5	630.5	2.51	0.2100	0.202	0.28
“ phenylhydantoin			2.48			
Aspartic acid	111.2	270.0	4.27	0.1463	0.1223	0.17
“ “ phenylhydantoin			3.98			
Glutamic acid	171.0	278.2	6.94	0.1820	0.2109	0.29
“ “ hydrochloride			6.86			
Proline (as rhodanilate)	42.8	212.0	0.522	0.031	0.0115	0.01
“ phenylhydantoin			0.501			
Hydroxyproline	0.51†	223.5	1.053	4.64	0.0180	0.02
Glycine‡	121.2			0.0293†	0.0469	0.06
Serine‡	104.0			0.0027†	0.0026	

\* Values taken from Block and Bolling (17)

† Assayed by method of Troll and Cannan (14)

‡ Isolated carrier-free

must have followed a fairly direct pathway from hydroxyproline. Practically no activity was located in the carboxyl carbon, hence all the activity was presumably still in the  $\alpha$ -carbon, as in the injected hydroxyproline. By comparison, a degradation of glycine isolated from liver protein (Table IV) showed almost equal distribution of activity between the carboxyl and methylene carbons, being probably derived from alanine by an indirect pathway, a conclusion also supported by its low level of activity. It should be noted that the activity and specific activity of serine were lower than that of glycine (Table III), possibly indicating that glycine is formed from alanine by a pathway not involving serine.

The formation of alanine from hydroxyproline is envisaged as being analogous to the formation of glycine and acetaldehyde from threonine, another hydroxyamino acid (15)  $\gamma$ -Hydroxyglutamic acid or a related

TABLE IV

*Degradation of Alanine and Glycine*

Samples from liver protein of rat dosed with DL-hydroxyproline, diluted with carrier (see Table III)

	Specific activity
	$\mu\text{c per mmole} \times 10^{-3}$
Alanine	25 13
CO <sub>2</sub> from carboxyl	1 21 (4 8%)
Acetaldehyde dinitrophenylhydrazine from carbon chain	25 01 (99 4%)
Glycine	1 83
CO <sub>2</sub> from carboxyl	0 81 (44 3%)
Formaldehyde dimedon derivative from methylene	0 90 (49 2%)

TABLE V

*Amino Acids from Liver Protein*

The dose injected was 73 8  $\mu\text{c}$  of DL-allohydroxyproline, specific activity, 664  $\mu\text{c}$  per millimole, liver protein, dry weight, 1 496 gm, duration of experiment, 3 hours and 50 minutes

Amino acids and derivatives	Amount present*	Amount of carrier added	Specific activity of diluted compound	Calculated specific activity of undiluted amino acid	Total activity of amino acid in protein	Per cent of dose
	mg	mg	$\mu\text{c per mmole} \times 10^{-3}$	$\mu\text{c per mmole}$	$\mu\text{c}$	
Aspartic acid	116 8	180 0	3 95	0 1002	0 088	0 12
Glutamic "	179 5	435 0	2 66	0 0911	0 111	0 15
Proline (as rhodanilate)	44 8	178 2	0 33	0 0165	0 006	0 01
Hydroxyproline	0 54†	177 4	1 58	5 20	0 021	0 03
Alanine‡	89 8			0 0371†	0 037	0 05

\* Values taken from Block and Bolling (17)

† Assayed by method of Troll and Cannan (14)

‡ Isolated carrier-free

compound may result from opening of the hydroxyproline ring and could then split into alanine and glyoxylic acid or a related 2-carbon aldehyde. This hypothesis could be proved by the use of hydroxyproline labeled in the carbon which bears the hydroxyl group.

Appreciable activity was found in aspartic and glutamic acids (Tables III and V). Degradation experiments (Table VI) showed that, whereas

aspartic acid had over 70 per cent of its activity in the methylene carbons, presumably in the  $\alpha$ -carbon, glutamic acid had only about one-third of its activity in the  $\alpha$ -carbon, most of it being in the  $\beta$ - or  $\gamma$ -carbons, or both. These results exclude a pathway whereby aspartic acid is derived from glutamic acid. In such a case (6), the proportion of labeling in the  $\alpha$ -carbon and the  $\beta$ - and  $\gamma$ -carbons of glutamic acid is almost identical with that of the carboxyls and methylenes, respectively, of aspartic acid. Nor can there be a direct and independent pathway from hydroxyproline to glutamic acid, because of the lower activity of its  $\alpha$ -carbon, compared to its

TABLE VI

*Degradation of Aspartic and Glutamic Acids*

Samples from liver protein of rat dosed with DL-hydroxyproline, diluted with carrier (see Table III)

	Specific activity
	$\mu\text{c per mmole} \times 10^{-3}$
Aspartic acid	42.7
(CO <sub>2</sub> from carboxyls of aspartic acid) $\times 2$	12.0 (28.1%)
Methylene carbons of aspartic acid, calculated	30.7 (71.9%)
Glutamic acid	56.2
Succinic " from glutamic acid	47.8 (85.0%)
$\alpha$ -Carboxyl of glutamic acid, calculated	8.4 (14.9%)
(CO <sub>2</sub> from carboxyls of succinic acid) $\times 2$ (representing $\alpha$ -carbon and $\gamma$ -carboxyl of glutamic acid)	19.1 (34.0%)
Methylene carbons of succinic acid (representing $\beta$ - and $\gamma$ -carbons of glutamic acid), calculated	28.8 (51.3%)

$\beta$ - and  $\gamma$ -carbons. The results are compatible with, though they do not prove, a derivation of glutamic from aspartic acid. It is further postulated that aspartic acid is formed by a separate, second pathway from hydroxyproline and not through alanine. The alanine of the animal receiving allohydroxyproline had very low activity, whereas the radioactivity of its aspartic acid was still high (Table V). It appears that, in the metabolism of allohydroxyproline, alanine is not produced, and only the second pathway to aspartic acid is functioning.

Womack and Rose (4) found that, whereas glutamic acid and proline could, hydroxyproline could not partially replace arginine in the diet of rats deprived of arginine, proline, and glutamic acid. If it is assumed that glutamic acid is an intermediate between proline and arginine, then the



present results are not in accord with those of Womack and Rose, since hydroxyproline is transformed into glutamic acid. However, if it is postulated that proline is changed directly to ornithine, which is then used in arginine synthesis, and glutamic acid is only a secondary product (*cf* (16)), then one could explain the lack of arginine formation from hydroxyproline. The metabolism of hydroxyproline would then differ from that of proline by the absence of a pathway to ornithine. Its pathway to glutamic acid would be indirect and therefore slow. In fact, no significant amount of radioactivity was detected in the basic amino acids, which included arginine.

The low activity in proline (Tables III and V) confirms the results of Stetten (3) obtained with  $N^{15}$ -hydroxyproline, and signifies the absence of a direct reduction reaction of hydroxyproline *in vivo* to give proline. The activity found in proline is, no doubt, derived through glutamic acid.

The presence in liver protein of hydroxyproline, and in amounts in excess of direct incorporation of injected radioactive hydroxyproline (specific activity,  $4.64 \mu\text{c}$  per millimole, specific activity of injected hydroxyproline,  $664 \mu\text{c}$  per millimole, level, 0.036 per cent of dry weight), is here demonstrated for the first time. These data are consistent with the findings of Troll and Cannan (14), who showed the presence of 0.005 per cent hydroxyproline in serum albumin. It appears that proteins other than collagen can have low levels of hydroxyproline.

Muscle and tendon protein were analyzed for radioactivity. However, because of the low turnover of these proteins and the short duration of the experiment, incorporation was insignificantly small.

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#### SUMMARY

1. DL-Hydroxyproline- $\alpha$ - $C^{14}$  and DL-allohydroxyproline- $\alpha$ - $C^{14}$  were synthesized.

2. After injection into rats, a high level of activity was obtained in the expired carbon dioxide of the animal receiving natural hydroxyproline, and about one-half the amount in the animal receiving the allo isomer.

3. One principal liver and one principal urinary metabolite were found, different from each other and different both from  $\gamma$ -hydroxyornithine and  $\gamma$ -hydroxyglutamic acid.

4. Urinary hydroxyproline had a specific activity only slightly less than injected hydroxyproline, suggesting the existence of a very small or non-existent free hydroxyproline pool, though some bound hydroxyproline could be detected in liver protein.

5 The principal labeled amino acid in the liver protein of the animal receiving natural hydroxyproline was alanine, with glutamic and aspartic acids following closely. In the animal which received the allo isomer, glutamic and aspartic acids had the highest activity. Proline, hydroxyproline, glycine, and serine had some, though little, activity. No other radioactive amino acid could be detected.

6 Degradation studies revealed that alanine was derived fairly directly from hydroxyproline. The results suggest that there exists a second pathway to aspartic acid, which then gives rise to glutamic acid.

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# UREA AS A SOURCE OF NITROGEN FOR THE BIOSYNTHESIS OF AMINO ACIDS\*

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Urea has long been recognized as the principal end product of nitrogenous metabolism in mammals. In recent years, the possibility that monogastric animals might be able to decompose this substance, when administered preformed, has stimulated considerable interest. The presence of radioactive carbon dioxide in the expired air after the injection of urea labeled with  $C^{14}$  indicates that decomposition of this compound actually occurs in the mouse (2) and the rat (3), and to a slight extent in the cat (4). However, attempts to determine whether the rat, as a typical non-ruminant, can utilize the nitrogen of urea for synthetic purposes have yielded divergent conclusions. Thus, Kriss and Marcy (5) found that urea administered orally to immature rats receiving a presumably normal diet exerted no change in body weight and was almost quantitatively recovered as such in the urine and feces. Similar results have been reported by Bloch (6), following experiments upon mature rats. This investigator added urea which had been labeled with  $N^{15}$  to a diet containing casein, and observed the extent of excretion of the compound and the degree of incorporation of the isotope in certain tissue constituents. The findings led him to conclude that urea is devoid of any metabolic activity.

The above data are in striking contrast to those set forth in a previous paper from this laboratory by Rose, Smith, Womack, and Shane (7). The latter were investigating the types of compounds which might serve as sources of nitrogen for the synthesis of the non-essential amino acids. For this purpose, advantage was taken of the fact that the growth of weanling rats is markedly inhibited by restricting the dietary nitrogen to that present in the essential amino acids when each is furnished at the minimal level.

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compatible with maximal growth (8-10) It should be recalled that these minimal values were established under conditions which provided an abundance of all amino acids other than the one under investigation (*cf* (8)) Consequently, one would anticipate that a diet carrying the essential amino acids only, each at its minimal level, would be incapable of supporting normal gains in weight, since, under these circumstances, sufficient nitrogen would not be available for the synthesis of the non-essential acids In other words, under the conditions specified, a shortage of nitrogen might become the limiting factor in the growth of the subjects Experience showed this supposition to be correct, but of greater significance for the problem at hand was the disclosure that the growth of the animals could be conspicuously accelerated by the addition to the basal ration of any one of several nitrogenous compounds, notably ammonium salts, L-glutamic acid, glycine, and even urea These findings, which have been confirmed elsewhere (11), appear to permit of only one reasonable interpretation, namely that the nitrogen of the dietary supplements was utilized in the synthesis of amino acids

It should be emphasized that the diets employed in the experiments of Kriss and Marcy (5) and Bloch (6) were "normal" with respect to their nitrogen content, and may be presumed to have furnished all of the amino acids On the other hand, as pointed out above, the growth tests conducted in this laboratory (7) involved the use of a ration which was devoid of the non-essential amino acids and carried only minimal quantities of the essentials In view of this fundamental difference, it seems plausible to suspect that the character of the nitrogen intake may have been responsible for the divergence between our findings and those of others To test this hypothesis, a comparison was made of the effects of adding urea to rations in which the nitrogen was supplied in the form of (a) casein, and (b) a mixture of the essential amino acids at their minimal levels Furthermore, the urea was labeled with  $N^{15}$  in order thereby to permit direct measurements of the extent of incorporation of the isotope in the amino acids of the tissues The conduct of the experiments and the results obtained are outlined below The data serve to substantiate the conclusions reached by the growth technique and to provide a reasonable explanation for the negative findings of others

#### EXPERIMENTAL

*Preparation of Labeled Urea*—The starting material in the preparation of labeled urea was ammonium nitrate containing 63.5 atom per cent excess of  $N^{15}$  in the ammonium ion<sup>1</sup> The salt was converted into ammonium chloride by treatment with strong alkali and distillation into an excess of

<sup>1</sup> Purchased from the Eastman Kodak Company, Rochester, New York

standard hydrochloric acid. The apparatus used for this purpose was similar to that described by Schoenheimer and Ratner (12). During the distillation, a slow stream of washed nitrogen was passed through the system to facilitate the transport of the ammonia. The resulting solution of ammonium chloride was then treated with a slight excess of a suspension of freshly prepared silver cyanate. The flask containing the mixture was protected from light and agitated for 6 hours on a mechanical shaker. After removing the silver salts by filtration, the combined filtrate and washings were slowly evaporated to dryness over a steam cone to accomplish the Wohler rearrangement of the isotopic ammonium cyanate into urea. Repeated extraction of the residue with 95 per cent ethanol removed the urea and left behind a grayish material derived from the slight excess of silver cyanate. The urea was purified by recrystallization from hot, absolute ethanol. Finally, it was recrystallized with an appropriate amount of previously analyzed unlabeled urea to yield a product containing the desired level of isotope. The preparation as used in the animal experiments contained 14.75 atom per cent excess of  $N^{15}$ . It melted sharply at  $132-133^{\circ}$  (uncorrected) and showed a total nitrogen content which agreed closely with the theoretical value.

(1)  $CH_4N_2O$  Calculated, N 46.91, found, N 46.88<sup>2</sup>

The purity of the labeled urea was further demonstrated by comparing its isotopic content with that of a sample of urea oxalate prepared from it.

*Feeding Experiments*—Six male, weanling rats of the same litter served as the experimental subjects. At the start of the feeding tests, they were quite uniform in size and had an average body weight of 45 gm. They were divided as equitably as possible into two groups of three rats each. Hereafter, they will be referred to as the animals of Group A, which received Diet A containing the essential amino acids, and the animals of Group C, which received Diet C containing casein. Each rat was housed in a separate cage, which was designed to prevent food scattering and to permit quantitative collection of the urine and feces.

The make-up of the basal diets is shown in Table I. As will be observed, Diet A contained amino acid Mixture XXVI, which was the mixture used by Rose *et al.* (7) in demonstrating the growth-stimulatory effect of added urea. Diet C was a normal ration. It carried 18 per cent of casein and 0.20 per cent of DL-methionine. In our experience, the addition of methionine improves the quality of casein and permits slightly better growth. Both diets were supplemented with suitable quantities of vitamins, as described elsewhere (*cf.* (10)).

Throughout the entire experiment, the rats of Group A were permitted

<sup>2</sup> Values are corrected for 14.75 atom per cent excess of  $N^{15}$ .

to consume food *ad libitum*. During the first 12 days, the basal ration (Diet A) alone was administered. At the expiration of this fore period, the labeled urea was incorporated in the ration in the proportion of 1.23 per cent and at the expense of an equal weight of dextrin. The labeled urea furnished 0.577 gm of additional nitrogen per 100 gm of Diet A. This is comparable to the nitrogen content (0.574 gm) of the non-isotopic urea previously found to be effective in the stimulation of growth (7). A total of 176 gm of Diet A was supplemented with labeled urea, and the

TABLE I  
*Composition of Basal Diets*

Component	Diet A	Diet C
	<i>gm</i>	<i>gm</i>
Amino acid Mixture XXVI*	8.82	
Casein		18.00
DL-Methionine		0.20
Sucrose	15.00	15.00
Dextrin	67.43	58.05
Cellu flour	2.00	2.00
Salt mixture†	4.00	4.00
Corn oil	2.00	2.00
Haliver oil‡	0.05	0.05
Inositol	0.10	0.10
Choline chloride	0.20	0.20
Liver extract§	0.40	0.40
	100.00	100.00

\* Rose *et al* (7)

† Jones and Foster (13)

‡ This contained 65,000 U S P units of vitamin A and 13,000 U S P units of vitamin D per gm

§ Wilson's liver powder, 1.20

excess N<sup>15</sup> therein contained was 158.8 mg. The food was kept constantly before the animals of Group A until it was entirely consumed, care being taken to note the exact time when the last trace disappeared. This occurred in 7½ days.

The rats of Group C were allowed to consume the casein diet (Diet C) *ad libitum* for 8 days. As was to be anticipated, they made rapid gains in weight, consequently, the paired feeding technique was instituted on the 9th day and was continued to the end of the experiment. According to this procedure, the food intake of the animals of Group C was limited to the voluntary consumption of the rats of Group A. Since all animals were weighed and their food intakes determined at 4 day intervals, use of the

paired feeding technique necessitated an extension of the fore period of Group C by 4 days in order that an accurate estimate of the consumption of Group A during the preceding 4 days might be established. After 16 days, 1.23 per cent of isotopic urea was incorporated in 176 gm of Diet C, at the expense of dextrin, and the resulting supplemented ration was administered for exactly  $7\frac{1}{3}$  days. To equalize, as nearly as possible, the conditions to which the two groups of animals were being subjected, the daily allotment of Diet C was furnished to each member of Group C in three equal portions at 8 hour intervals. Thus, during the period of urea supplementation, the rats of each group received exactly the same weight of food and of isotopic urea.

Beginning with the addition of urea to the diets, the urines were collected under toluene. At frequent intervals, the funnels through which the urines passed to the receiving vessels were washed down with a fine stream of 2 per cent boric acid solution. Each sample was strained through a wad of cotton prior to storing. At the conclusion of the feeding tests, the urines from the three animals of each group were combined and reserved at a low temperature for analysis. The feces were collected several times daily, covered with acidified ethanol, and evaporated to dryness. At the end of the period, the combined fecal material from the three rats of each group was ground to a fine powder and saved for analysis.

The animals were killed by decapitation immediately after they had completed the consumption of the supplemented diets. The gastrointestinal tracts were first excised. Formed pellets of fecal material were recovered and added to the appropriate samples previously collected. The remainder of the alimentary contents was removed by thorough washing and the carcasses of the three members of each group were combined for subsequent work. For the latter purpose, they were cut into small pieces, frozen, and passed through a meat grinder. Each of the resulting mixed tissues was homogenized in a Waring blender with two 500 ml portions of 10 per cent trichloroacetic acid solution. After filtration, the residues were extracted twice with 300 ml portions of acetone and repeatedly with ether. When dried in air, fluffy powders resulted, which consisted predominantly of the proteins and part of the inorganic components of the animals.

*Isolation and Analytical Procedures*—Portions of the carcass powders described above were used in ascertaining their total and isotopic nitrogen content. The methods employed will be outlined later. Larger samples were used in isolating individual amino acids. From each, one essential and several non-essential amino acids were procured. Urea was obtained from each of the combined urines. All components were analyzed for their  $N^{15}$  content. Attention is called to the fact that in no instance was a carrier employed in the isolation procedure, consequently, the isotopic values



to be reported later represent those which actually existed in the compounds at the time the subjects were killed

For the isolation of amino acids, 30 and 40 gm of the carcass materials derived from the animals of Groups A and C, respectively, were hydrolyzed by refluxing for 23 hours with 300 ml of 6 N hydrochloric acid. Each hydrolysate was concentrated *in vacuo* to a syrup and freed from most of the excess hydrochloric acid by repeated addition of water and evaporation under reduced pressure. The final products were dissolved in water and filtered. The combined filtrate and washings from each preparation amounted to approximately 1000 ml. Each was adjusted in reaction to pH 3 and treated with charcoal (Darco G-60) for the removal of *tyrosine* and *phenylalanine*, as described by Partidge (14). The *tyrosine* was separated from the *phenylalanine* by means of its insolubility in cold water. Solution of the crude *tyrosine* in hot water, followed by decolorization, yielded the pure amino acid, as indicated by the accompanying analytical data. No attempt was made to recover the *phenylalanine*.

(2)	$C_9H_{11}O_3N$	Calculated	C 59.66, H 6.12, N 7.73
		Found (Group A)	" 59.72, " 6.21, " 7.84
		" ( " C)	" 60.09, " 6.08, " 7.78

*Cystine* was isolated from the filtrates remaining after the removal of the aromatic amino acids. For this purpose, each solution was concentrated *in vacuo* to approximately 200 ml, adjusted to pH 5.5, and allowed to stand for several days in the cold room. The crude *cystine* so obtained was repeatedly crystallized until pure by dissolving in hydrochloric acid and adding solid sodium acetate until a negative test for mineral acid was given by Congo red paper.

(3)	$C_6H_{12}O_4N_2S_2$	Calculated	C 29.99, H 5.03, N 11.66
		Found (Group A)	" 30.11, " 4.99, " 11.69
		" ( " C)	" 29.75, " 5.09, " 11.53

For the recovery of *aspartic* and *glutamic acids*, each of the solutions from which the *cystine* had been crystallized was diluted to 1 liter and stirred repeatedly with Amberlite IRA-400 in the hydroxyl phase. During this process, the pH was progressively raised from 5.5 to approximately 7.7. The dicarboxylic amino acids were eluted from the resin with 4 per cent hydrochloric acid, *glutamic acid* being recovered as the hydrochloride and *aspartic acid* as the copper salt. Free *glutamic acid* was obtained by dissolving the hydrochloride in water, adjusting the pH to 3.5 by the dropwise addition of pyridine, and treating the resulting solution with 3 to 4 volumes of ethanol. On standing overnight in the cold, *glutamic acid* was deposited and was removed by filtration. Purification was accomplished by reconversion into the hydrochloride, liberation of the free amino acid

as before, and crystallization from water and ethanol. Analyses of the final products are shown below

(4)	$C_5H_9O_4N$	Calculated	C 40.81, H 6.17, N 9.52
		Found (Group A)	" 40.57, " 5.94, " 9.43
		" ( " C)	" 41.02, " 6.14, " 9.41

The copper aspartate was dissolved in 1 N hydrochloric acid and decomposed with hydrogen sulfide. The filtrate and washings from the copper sulfide were concentrated *in vacuo* to a syrup and dissolved in 10 ml of warm water. The pH of the solution, which at this point was approximately 1.0, was raised to 3.5 by the addition of pyridine and the whole was treated with 3 to 4 volumes of ethanol. After standing overnight in the cold, the white crystals of aspartic acid were removed by filtration and recrystallized from aqueous ethanol until analytically pure.

(5)	$C_4H_7O_4N$	Calculated	C 36.09, H 5.30, N 10.52
		Found (Group A)	" 36.14, " 5.27, " 10.46
		" ( " C)	" 36.13, " 5.32, " 10.69

The four amino acids, the isolation of which has been outlined above, obviously are non-essential components of the food of the rat (15). For comparative purposes, it seemed desirable to obtain from the tissues an amino acid which, for the species in question, is a typical member of the group of indispensable dietary constituents. *Histidine* was chosen for this purpose. It was isolated from the hydrolysates remaining after the adsorption of glutamic and aspartic acids. The pH of these solutions had been raised to 7.7 incidental to the removal of the dicarboxylic amino acids. Each solution was treated as follows. The pH was first reduced to 5.5 by the cautious addition of hydrochloric acid, and the resulting fluid was slowly percolated through a column of Amberlite IRC-50 which previously had been buffered to pH 4.7 with an acetate-acetic acid mixture. After all of the hydrolysate had passed through the column, the effluent and washings were set aside for the isolation of proline.

The basic amino acids were then eluted from the resin by percolating a 4 per cent hydrochloric acid solution through the column until the eluate gave a negative ninhydrin test. The excess of hydrochloric acid was removed by concentration to dryness *in vacuo* three times, the residue being taken up successively in 500 ml portions of distilled water. The final solid was dissolved in 600 ml of water, and the whole was stirred with Amberlite IR-45, in the hydroxyl phase, until the pH had been raised to 6.6. The solution of basic amino acids was now passed through a second column of Amberlite IRC-50 which had been buffered to pH 7.0. At the latter pH, the resin adsorbs the lysine and arginine but permits the histidine to pass through the column. The effluent was then concentrated to a small

volume, filtered, and treated with 3,4-dichlorobenzenesulfonic acid under the conditions described by Vickery (16), and with a reagent prepared in accordance with his directions (17). After standing at a low temperature for several days, the crystalline histidine disulfonate was removed by filtration and converted into the monohydrochloride monohydrate. The latter salt was recrystallized until pure.

(6)	$C_6H_{12}O_3N_3Cl$	Calculated	C 34.37, H 5.77, N 20.05
		Found (Group A)	" 34.19, " 5.75, " 20.06
		" ( " C)	" 33.96, " 5.60, " 20.01

For the isolation of *proline*, the method of Bergmann (18) was employed. Unfortunately, it was successful only in the case of the protein hydrolysate obtained from the rats of Group A. An attempt was made to procure a comparable sample from the animals of Group C, but this met with failure. On other occasions in this laboratory, difficulty has been encountered with the Bergmann procedure. No entirely trustworthy method for the isolation of proline from small quantities of proteins appears to be available.

In the successful experiment, the effluent and washings from the first column of Amberlite IRC-50 were concentrated under reduced pressure to a small volume and treated with sufficient 6 N hydrochloric acid to render the solution approximately 0.25 N. An excess of ammonium rhodanilate in methanol was then added, and the resulting mixture was placed in the cold room for 2 days. The L-proline rhodanilate which separated was recrystallized from acidified methanol. To obtain the free amino acid, advantage was taken of the relative insolubility of pyridine rhodanilate in water, as suggested by Bergmann. Accordingly, the proline rhodanilate was suspended in 50 ml. of distilled water containing 3 ml. of reagent grade pyridine and agitated intermittently for several hours. After filtering the solution and washing the precipitate with cold water, a pink fluid was obtained. The chromogenic material was not removed by charcoal, therefore, the solution was taken to dryness by lyophilization, dissolved in water, and lyophilized again. The solid so obtained was repeatedly crystallized by dissolving it in absolute ethanol, filtering, and adding anhydrous ether. All but a trace of the color which remained was removed by extracting the solid with a small portion of ice-cold, absolute ethanol. Further crystallization of this product yielded analytically pure proline.

(7)	$C_5H_9O_2N$	Calculated	C 52.16, H 7.88, N 12.17
		Found (Group A)	" 51.72, " 7.84, " 12.19

All of the isolated amino acids described above were tested for homogeneity by paper chromatography. Each exhibited a single, discrete spot and an  $R_F$  value which was identical with that of a pure sample of the amino acid when the isolated compound and its control were chromatographed simultaneously on the same strip of filter paper.

In addition to the tissue amino acids, a sample of urea was isolated as its xanthhydiyl derivative from each of the mixed urines collected from the animals of Groups A and C. For this purpose, an appropriate aliquot of urine was decolorized with charcoal, acidified strongly with glacial acetic acid, and treated with a reagent consisting of xanthhydiol dissolved in a mixture of absolute methanol and glacial acetic acid. When precipitation was complete, the xanthhydiyl urea was removed by filtration through a sintered glass funnel, washed with 66 per cent acetic acid, and purified by crystallization from a dioxane-water mixture.

(8)	$C_{27}H_{20}O_3N_2$	Calculated	N 6.66
		Found (Group A)	" 6.57
		" ( " C)	" 6.64

In analyzing the compounds isolated from the tissues and urines, carbon and hydrogen were determined in the Microanalytical Laboratory of this Department. Total nitrogen was estimated by the micro-Kjeldahl procedure, a mixed catalyst composed of potassium sulfate, copper sulfate, and selenium dioxide being employed, and use being made of a prolonged digestion period to insure complete oxidation (*cf* Chibnall *et al* (19)). In measuring the isotopic nitrogen content, each sample was first digested as in the total nitrogen determinations, after which the resulting ammonia was converted into gaseous nitrogen by the action of an alkaline hypobromite solution. The latter step was carried out in a small reaction vessel to which was attached a tipping flask containing the hypobromite. Both containers were connected to a high vacuum system. After the system had been evacuated to  $10^{-5}$  mm of mercury, the hypobromite was tipped into the ammonia solution and heat was applied gently with a microburner. As the nitrogen gas was liberated, it passed through a U trap which was immersed in a liquid nitrogen bath to remove water vapor and any other condensable gases. As soon as the evolution of nitrogen had ceased, the gas was transferred to a spectrometer bulb, which was then disconnected from the vacuum line and attached to the mass spectrometer. The  $N^{15}$  analyses were performed with a Consolidated-Nier isotope ratio mass spectrometer, model No. 21-201. As a check against the possibility of contamination by air, all samples were routinely tested for the presence of molecular oxygen of mass 32. Contamination so indicated was well within the 3 per cent allowed by previous workers (20), falling as a rule between 0.5 and 1.0 per cent.

### Results

The findings in the two groups of animals are summarized in Tables II and III. In Table II is presented a partial  $N^{15}$  balance for each dietary regime. Since the carcass proteins and their component amino acids were

of primary interest for the purposes of this investigation, no attempt was made to recover all of the administered isotope. In comparable experiments involving the administration of  $N^{15}$ -labeled amino acids, others have shown that the non-protein nitrogenous constituents of the tissues may contain 7.8 to 11.7 per cent of the isotope (21, 22). The  $N^{15}$  content of these components was not determined in our tests. Furthermore, since the subjects were sacrificed immediately after they had consumed the last of the allotted food, some loss of isotopic urea must have occurred when the alimentary tracts were washed out. The lower percentage recovery of  $N^{15}$  in the animals of Group A, as contrasted with those of Group C, may have

TABLE II

*Partial Balance of  $N^{15}$  after Feeding Isotopic Urea*

Each group, composed of three weanling rats, consumed 2.165 gm of isotopic urea containing 158.8 mg of excess  $N^{15}$

	Group A				Group C			
	Total N content	$N^{15}$ content	Total $N^{15}$	$N^{15}$ recovered	Total N content	$N^{15}$ content	Total $N^{15}$	$N^{15}$ recovered
	gm	atoms per cent excess	mg	per cent	gm	atoms per cent excess	mg	per cent
Carcass protein	5.20	0.618	34.40	21.66	9.83	0.046	4.84	3.05
Excreta								
Urine	0.83	7.251	64.12	40.38	2.80	4.281	127.97	80.59
Feces	0.45	4.776	22.94	14.44	0.49	0.756	3.96	2.47
Total $N^{15}$ recovered			121.46	76.48			136.77	86.11

been due to the smaller sample of carcass protein which was available for analysis, since the rats receiving the amino acid diet gained less than did their litter mates upon the casein ration.

The data in Table II demonstrate clearly that the degree of tissue incorporation of urea nitrogen is much greater when the basal diet carries the essential amino acids only (Group A) than when all amino acids are furnished in the form of casein (Group C). Of the administered isotope, that found in the tissue proteins amounted to 21.66 and 3.05 per cent, respectively. In contrast to these findings, twice as much  $N^{15}$  was excreted in the urines by the animals of Group C as by those of Group A. These results were to be expected in view of the earlier observations of Rose *et al* (7) and of Bloch (6), to which reference has already been made. The figures confirm the conclusion that the nitrogen of urea can be utilized

when the subjects are in need of this element for synthetic purposes. No entirely satisfactory explanation can be offered to account for the much higher fecal output of the isotope by the members of Group A. Possibly, the explanation is to be found in the mild laxative action of diets containing mixtures of amino acids, thereby leading to more rapid passage of food through the alimentary tract and less complete absorption of the urea. Perhaps a more likely explanation is one suggested by Ratner *et al* (22) to account for the isotopic content of the feces following the administration of labeled glycine. These investigators believe that the isotope "entered the intestinal lumen with the proteins of the intestinal secretions, which, like the proteins and the other nitrogenous constituents of the animals, must have contained  $N^{15}$ ."

One would anticipate that, under the conditions of our experiments, the

TABLE III  
*N<sup>15</sup> Content of Isolated Products*

	Group A	Group C
	<i>atom per cent excess</i>	<i>atom per cent excess</i>
Tyrosine	0.542	0.023
Cystine	0.433	0.027
Glutamic acid	0.962	0.071
Aspartic "	0.841	0.051
Proline	0.552	
Histidine HCl H <sub>2</sub> O	0.103	0.009
Urea (urinary)	14.046	4.884

rats of Group A would need nitrogen primarily for the synthesis of the non-essential amino acids, and that a comparable need would not be experienced by the subjects of Group C. If this assumption is correct, the non-essential amino acids derived from the animals of Group A should be labeled more highly than the corresponding amino acids obtained from the members of Group C. This proved to be the case to an astonishing degree, as exemplified by the data in Table III. Omitting for the moment the values for tyrosine, one will observe that the figures representing the  $N^{15}$  content of the non-essential amino acids of Group A are quite high, indeed, they are 14 to 16 times the values given for the corresponding compounds obtained from Group C. The highest isotopic concentrations were observed in glutamic and aspartic acids. This is in keeping with the well known role of these amino acids in transamination reactions (*cf* (23)).

With respect to tyrosine, the high isotopic content of the sample from Group A was unexpected. An abundance of evidence is available which demonstrates that tyrosine has its origin in phenylalanine (24, 25), and that

the conversion of the latter into the former occurs despite the presence or absence of preformed tyrosine in the food (25) <sup>3</sup> In the present study, the ration of the animals of Group A was *devoid* of tyrosine, therefore, all of the tyrosine utilized by the organism for growth purposes must have been formed from phenylalanine Little is known concerning the mechanism involved in this reaction, although an enzyme system has been described which is capable of catalyzing the over-all oxidation (26) The fact that the tyrosine was so highly labeled with N<sup>15</sup> constitutes strong presumptive evidence against a *direct* conversion of the benzene ring of phenylalanine into the phenol ring of tyrosine In the light of our data, it appears much more reasonable to assume that deamination precedes oxidation, and that a phenolic intermediate undergoes reamination with the formation of tyrosine

In contrast to the isotopic levels observed in the non-essential amino acids, the values for the two samples of histidine (Table III) were much lower Since this amino acid is an indispensable dietary component for the rat (15), the only mechanism whereby N<sup>15</sup> could have entered the molecule is by a "continuous process of successive deamination and amination," as observed by Schoenheimer *et al* (27) following the administration of isotopically labeled ammonium citrate The latter compound was added to a diet containing 16 per cent of casein The histidine isolated from the carcass protein was shown to have an N<sup>15</sup> content of 0.013 atom per cent excess, all of which was located in the  $\alpha$ -amino group This value is comparable to that observed in the present investigation in the animals of Group C, which also received a normal casein ration On the other hand, the isotopic content of the histidine obtained from the rats of Group A, though decidedly lower than the levels present in the non-essential amino acids, was several times greater than that of the histidine derived from Group C Possibly, this latter fact may be accounted for by an intensification of the process of deamination and amination incidental to the simultaneous biosynthesis of all of the non-essential amino acids Indeed, a shift of nitrogen from one amino acid to another, or from and to the nitrogen "pool," must occur with remarkable facility inasmuch as Diet A (Table I), even without an additional source of nitrogen, permits slow growth (*cf* (7)) Since Diet A contained only the essential amino acids, and these at their minimal levels, gains in weight would have been impossible were it not for the use of some of the nitrogen of the essential acids in the biosynthesis of the non-essential acids This process should have afforded sufficient opportunity for the histidine derived from the animals of Group A to have become more heavily labeled than the histidine origi-

<sup>3</sup> Howe, E. E., and Rose, W. C., unpublished data (quoted in Moss and Schoenheimer (25))

nating in the rats of Group C, which were not confronted with a deficient supply of nitrogen

With respect to the urea samples isolated from the urines of the two groups of animals (Table III), little need be said. Obviously, the isotopic urea which was not used by the animals of Group A experienced very little dilution with non-isotopic urea before being excreted. The urinary sample contained 14.046 atom per cent excess of  $N^{15}$ , while the administered urea contained, it will be recalled, 14.75 atom per cent excess. Not more than a small decrease in isotopic content was to have been expected, since the physiologically active amino acids of Diet A furnished only 0.81 per cent of total nitrogen. On the other hand, the sample of urea derived from the rats of Group C was much lower in its isotopic content (4.884 atom per cent excess). Evidently, it had been diluted by the urea arising in the organism during the metabolism of the 18 per cent casein ration.

Finally, attention is called to the fact that the experiments described above offer no explanation of the mechanism whereby the nitrogen of urea is rendered available for the uses of the organism. Recent studies of this aspect of the problem indicate that in the mouse (28) and in the rat (3, 29) the urease activity of gastrointestinal microorganisms is largely or entirely responsible for the decomposition of urea. In the hope that the growth technique might throw more light on this point, Diet A was supplemented with urea and administered to two groups of rats, one group of which also received the mixture of antibiotic agents used by Dintzis and Hastings (28) in studying the short time fate of urea labeled with  $C^{14}$ . At frequent intervals, fecal samples were obtained from our animals and subjected to bacterial counts.<sup>4</sup> Unfortunately, convincing growth data could not be obtained. The bacterial counts dropped promptly, as reported by Dintzis and Hastings, but rose again within a few days as the microorganisms acquired resistance to the drugs. This eventuality defeated the purpose of the experiments and rendered the findings inconclusive.

#### SUMMARY

Urea labeled with  $N^{15}$  has been administered to two groups of growing rats which were maintained on diets containing, respectively, (a) a mixture of the essential amino acids at their minimal levels (Group A), and (b) a normal ration containing 18 per cent of casein (Group C). The distribution of the isotope in the excreta and carcass proteins indicated an ex-

<sup>4</sup> We are deeply indebted to Dr. A. B. Hastings and Dr. R. Z. Dintzis for giving us full information concerning the composition of the media used by them in making the bacterial counts, and for other useful information. We are also grateful to Dr. I. C. Gunsalus for helpful advice regarding the bacteriological tests and to Mr. John R. Stamer of the Department of Bacteriology, University of Illinois, who actually made the counts.



tensive utilization of the urea nitrogen by the subjects of Group A, but not by those of Group C. Cystine, glutamic acid, and aspartic acid isolated from the animals of the first group contained the isotope in high concentrations. On the other hand, histidine, an essential amino acid for the rat, had a low level of  $N^{15}$ . The data are believed to provide unequivocal proof that the nitrogen of urea can be utilized for the synthesis of the non-essential amino acids when the latter are excluded from the food, and when no other source of nitrogen is available for the purposes in question.

Tyrosine isolated from the animals of Group A, like the other non-essential amino acids, was found to contain a high level of  $N^{15}$ . This amino acid, under the conditions of the experiment, must have originated in the phenylalanine of the ration. The high isotopic content is believed to provide strong presumptive evidence against a *direct* oxidation in the para position and to suggest that deamination of the amino acid must precede the conversion of the benzene group into the phenolic group.

The probable role of bacteria in the utilization of urea nitrogen is discussed.

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# ENZYMATIC FORMATION OF ASCORBIC ACID IN RAT LIVER EXTRACTS\*

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The investigations of King and his colleagues, carried out with the aid of isotopically labeled precursors, have established that ascorbic acid is formed from glucose in the intact rat largely by a mechanism in which the carbon chain of glucose remains intact (1). Furthermore, they have revealed that carbon atom 1 of D-glucose is the precursor of carbon atom 6 of the L-ascorbic acid (2) and, conversely, that carbon atom 6 of D-glucose is the precursor of carbon atom 1 of ascorbic acid (3). Further experiments with labeled D-glucuronolactone revealed that this substance is a more immediate precursor of ascorbic acid than is D-glucose (4). These important findings are supported by the investigations of Isherwood and his colleagues (5-7), who have demonstrated, in non-isotopic experiments, that net formation of ascorbic acid in cress seedlings and in intact rats is increased upon administration of D-glucuronolactone, D-galacturonolactone, L-gulonolactone, and L-galactonolactone. These findings, together with other considerations, led them to suggest the existence of two parallel pathways for the biosynthesis of ascorbic acid, which are identical in mechanism but start with different isomeric hexose precursors

(1) D-Glucose  $\rightarrow$  D-glucuronolactone  $\rightarrow$  L-gulonolactone  $\rightarrow$  L-ascorbic acid

(2) D-Galactose  $\rightarrow$  D-galacturonolactone  $\rightarrow$  L-galactonolactone  $\rightarrow$  L-ascorbic acid

With this information available, it appeared feasible to approach experimentally the detailed mechanism of formation of ascorbic acid in animal tissues with the use of cell-free preparations. This paper describes the enzymatic formation of ascorbic acid from D-glucuronic acid or its lactone in cell-free extracts and fractions of rat liver, as well as certain gross features of the reaction mechanism.

## EXPERIMENTAL

*Methods and Materials*—"Total" ascorbic acid, defined as the sum of L-ascorbic acid, dehydroascorbic acid, and 2,3-diketogulonic acid, was determined by the 2,4-dinitrophenylhydrazine method of Roe and Kuether (8).

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as modified by Geschwind, Williams, and Li (9) Ascorbic acid, *z e* the reduced form alone, was determined by reaction with 2,6-dichlorophenol-indophenol as described by Harris and Olliver (10) The rat liver extract used in most of the experiments was prepared by homogenizing the livers from Wistar or occasionally Sprague-Dawley rats with 2.5 volumes of 0.15 M KCl in a Potter-Elvehjem homogenizer The homogenate was centrifuged at  $3000 \times g$  to remove the nuclei and mitochondria, which are essentially completely agglutinated in the isotonic KCl The supernatant fluid is referred to in the text as "rat liver extract" The entire preparation was carried out at  $0^\circ$  and the extract generally used with minimal delay Solutions of L-ascorbic acid and all other unstable compounds used in the various experiments were freshly made and carefully neutralized just before addition to the enzyme reaction media

*Formation and Destruction of Ascorbic Acid*—In preliminary experiments designed to detect ascorbic acid synthesis by liver homogenates, the formation of the sum of ascorbic acid, dehydroascorbic acid, and 2,3-diketogulonic acid ("total" ascorbic acid) was determined rather than L-ascorbic acid specifically, since it is known that the latter acid is readily oxidized by animal tissues to dehydroascorbic acid, which in turn spontaneously hydrolyzes to 2,3-diketogulonic acid, itself an unstable compound The rate of oxidation of L-ascorbic acid in this manner could conceivably outstrip the rate of synthesis in tissue extracts In such preliminary experiments it was found that whole liver homogenates supplemented with D-glucuronolactone caused the net formation of small amounts of "total" ascorbic acid, despite a considerable ability to oxidize added L-ascorbic acid Further study, upon refinement of the experimental conditions, revealed that this activity resided entirely in the supernatant fluid remaining after removal of the mitochondria and nuclei by centrifugation It was also found that addition of ATP,<sup>1</sup> DPN,  $Mg^{++}$ , and nicotinamide increased the rate of formation of ascorbic acid Under these conditions net synthesis of "total" ascorbic acid by the rat liver extract was readily observable in the presence of D-glucuronolactone as precursor, in the absence of the latter, no detectable formation of ascorbic acid occurred (see Table I) Also included in Table I are data of parallel experiments which show the rate of destruction of added L-ascorbic acid over the same time interval It is seen that a large fraction of the small amounts of ascorbic acid added disappeared during the incubation For this reason it appears that the true

<sup>1</sup> Abbreviations ATP, adenosine triphosphate, DPN and TPN, di- and triphosphopyridine nucleotide, respectively, UTP and UDP, uridine tri- and diphosphate, respectively, Tris, tris(hydroxymethyl)aminomethane, DPNH, reduced diphosphopyridine nucleotide,  $DPN^+$ , oxidized diphosphopyridine nucleotide, TPNH, reduced triphosphopyridine nucleotide,  $TPN^+$ , oxidized triphosphopyridine nucleotide, ITP, inosine triphosphate, UDPG, uridine diphosphoglucose

amount of new "total" ascorbic acid synthesized in the experiments with D-glucuronolactone as substrate may be considerably greater than the net accumulations measured. Because of the magnitude of the rate of destruction of added L-ascorbic acid observed in such control experiments, they were usually performed as a routine check in all tests of ascorbic acid synthesis.

From many control experiments carried out in the absence of D-glucuronolactone (Table I), the impression has been gained that the "total" ascorbic acid value contributed by the liver extract alone, indicated by the zero time values, may not be identical with free ascorbic acid, since it dis-

TABLE I  
*Enzymatic Formation and Destruction of Ascorbic Acid*

The test system contained final concentrations of 0.02 M phosphate buffer, pH 7.4, 0.0016 M DPN, 0.0016 M ATP, 0.03 M nicotinamide, 0.004 M MgCl<sub>2</sub>, substrates in amounts listed below, and 0.7 ml of rat liver extract in a total volume of 2.5 ml. Incubated 2 hours at 37° in air.

Experiment No	Substrate added	Amount, $\mu$ moles	"Total" ascorbic acid, $\mu$ moles		
			Zero time	After incubation	Net change
1	D-Glucuronolactone	10	0.40	1.30	+0.90
	L-Ascorbic acid	0.67	1.10	0.57	-0.53
	None		0.43	0.40	-0.03
2	D-Glucuronolactone	10	0.47	1.11	+0.64
	L-Ascorbic acid	0.67	1.08	0.48	-0.60
	None		0.41	0.33	-0.08
3	D-Glucuronolactone	10	0.50	1.17	+0.67
	L-Ascorbic acid	1.0	1.49	0.89	-0.60
	None		0.49	0.43	-0.06

appears during incubation at a lower rate than added L-ascorbic acid. About 25 per cent of the endogenous ascorbic acid in the liver extract was found to be non-dialyzable, in agreement with the findings of Sumerwell and Sealock (11) on whole pork liver. Such a "bound" form of ascorbic acid (see also Jeffay (12)) may of course have quite different susceptibility to enzymatic attack from that of free ascorbic acid. The possible presence of chromogens other than free or bound ascorbic acid in the endogenous fraction has not been entirely excluded.

By means of the 2,6-dichlorophenol-indophenol titration, it was found that the "total" ascorbic acid formed enzymatically consisted largely of "true" ascorbic acid (over 80 per cent), presumably the remaining 20 per cent represented the sum of dehydroascorbic acid and 2,3-diketogulonic acid (Table II). Ascorbic acid was identified as the major reaction product

with certainty by paper chromatography of the concentrated trichloroacetic acid filtrate of the enzymatic reaction medium by the method of Chen, Isherwood, and Mapson (13). Readily visualized spots with  $R_F$  values identical with that of authentic L-ascorbic acid were observed, a faint spot corresponding to dehydroascorbic acid was also seen.

In Fig. 1 are presented data on the course of ascorbic acid formation and

TABLE II  
*Formation of "True" Ascorbic Acid*

The conditions were exactly as in Table I. The figures represent the net formation of "total" ascorbic acid (the sum of ascorbic acid, dehydroascorbic acid, and 2,3-diketogulonic acid, measured as 2,4-dinitrophenylhydrazones) and "true" ascorbic acid (the reduced form, measured by titration with 2,6-dichlorophenol-indophenol).

Substrate added	Amount, $\mu$ moles	Net "total" ascorbic acid formed, $\mu$ moles	Net "true" ascorbic acid formed, $\mu$ moles
D-Glucuronolactone	10	1.42	1.18
"	10	1.43	1.15

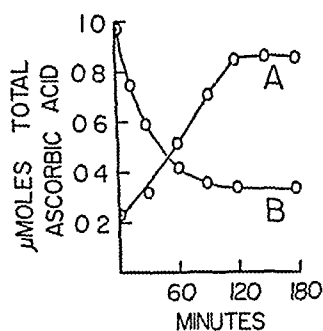


FIG. 1 Rate of formation and destruction of ascorbic acid. The test system was arranged exactly as in the experiments described in Table I. Curve A represents the rate of formation of "total" ascorbic acid from D-glucuronolactone, Curve B, the rate of disappearance of "total" ascorbic acid following addition of L-ascorbic acid.

destruction with time. Formation of total ascorbic acid proceeded linearly with time, reaching a plateau value at about 2 hours. Added L-ascorbic acid, however, rapidly disappeared at an *initial* rate which sometimes exceeded the rate of net synthesis. It is thus probable that the rate of formation measured is actually the result of a much higher absolute rate of formation and the considerable rate of destruction shown. However, it is not known whether part or all of the ascorbic acid formed enzymatically is generated in a bound form which may not be as susceptible as free ascorbic acid to enzymatic or non-enzymatic destruction.

*Cofactor Requirements*—The data summarized in Table III demonstrate that the formation of ascorbic acid from D-glucuronolactone in rat liver extracts proceeds at a maximal rate when ATP, DPN,  $Mg^{++}$ , and nicotinamide are present in the reaction medium. Omission of any one of these components caused a significant decrease in rate of synthesis in undialyzed extracts. The requirements for ATP, DPN, and  $Mg^{++}$  were more clearly demonstrated if the rat liver extract was first dialyzed against 0.15 M KCl at 0° for 7 hours with stirring (Table III).

TABLE III

*Components Required for Formation of Ascorbic Acid*

The complete test system contained 0.02 M phosphate buffer, pH 7.4, 0.0016 M DPN, 0.0016 M ATP, 0.03 M nicotinamide, 0.004 M  $MgCl_2$ , 0.004 M D-glucuronolactone, and 0.7 ml of enzyme in total volume of 2.50 ml. The reaction components were omitted as indicated below. In Experiment 3, 1  $\mu$ mole of UTP and 10  $\mu$ moles of glucose were added as shown. Incubated 2 hours at 37°.

Experiment No	Enzyme preparation	System	"Total" ascorbic acid formed, $\mu$ mole
1	Undialyzed rat liver extract	Complete	0.62
		DPN omitted	0.48
		ATP     "	0.34
		$Mg^{++}$ "	0.38
		Nicotinamide omitted	0.17
2	Dialyzed rat liver extract	Complete	0.26
		DPN omitted	0.01
		ATP     "	0.03
		$Mg^{++}$ "	0.17
		Nicotinamide omitted	0.24
3	Undialyzed liver extract	Complete	0.90
		"     + UTP	0.57
		"     +     "     and glucose	0.90

Still another cofactor requirement became evident on further study. If the undialyzed rat liver extract was permitted to stand at 3° for 20 to 30 hours, most of the synthetic activity was lost, despite addition of ATP, DPN, nicotinamide, and  $Mg^{++}$ . However, it was found that addition of TPN to the otherwise complete system could restore 50 per cent or more of the lost activity (Table IV). No other cofactor tested, including inosine triphosphate, UTP, coenzyme A, uridine diphosphoglucose, glucose-1-phosphate, and glucose-6-phosphate, was able to replace TPN. It appeared possible that TPN could replace the requirement for ATP plus DPN. The results of appropriate tests were equivocal, in dialyzed extracts, only partial replacement of ATP and DPN by TPN could be observed.



The formation of ascorbic acid occurred at a somewhat higher rate in phosphate buffer than in Tris buffer, but it was not possible to establish unequivocally a specific phosphate requirement in the crude extracts studied.

Since the amount of total ascorbic acid formed in these experiments is apparently the result of net synthesis and a rather substantial rate of breakdown, it appeared possible that the destruction of ascorbic acid might depend on one or more of the components observed to be required for net synthesis of ascorbic acid. However, the rate of destruction of added

TABLE IV  
*Requirement of TPN*

The standard test system contained 0.02 M phosphate buffer, pH 7.4, 0.0016 M DPN, 0.0016 M ATP, 0.03 M nicotinamide, 0.004 M  $MgCl_2$ , 0.004 M D-glucuronolactone, and 0.7 ml of liver extract, as indicated, in a total volume of 2.50 ml. Incubated 2 hours at 37°. The rat liver extract was inactivated by aging at 3° for 20 hours in Experiment 1 and 48 hours in Experiment 2.

Experiment No	Liver extract	Additions to standard test system	"Total" ascorbic acid formed, $\mu$ moles
1	Fresh	None	1.10
	Aged	"	0.06
	"	3.5 $\mu$ moles TPN	0.50
	"	0.7 " ITP	0.03
	"	0.7 " coenzyme A	0.05
	"	3.5 " UDPG	0.00
	"	3.5 " UTP	0.04
	"	3.5 " glucose-1-phosphate	0.03
2	Fresh	None	0.51
	"	6 $\mu$ moles TPN	0.48
	Aged	None	0.00
	"	6 $\mu$ moles TPN	0.33

L-ascorbic acid in the undialyzed rat liver extract was not affected by systematic omission of ATP, DPN, and  $Mg^{++}$ .

Although uridine nucleotides are known to be involved in the formation of glucuronic acid in animal tissues (14, 15), no evidence of a requirement or a stimulating effect of UTP, UDP, or uridine diphosphoglucose in ascorbic acid formation was observed. Actually, UTP was found to inhibit formation of ascorbic acid rather specifically, this inhibition was relieved by addition of D-glucose (Table III).

*Conversion of L-Gulonic Acid to Ascorbic Acid*—In order to determine the nature of the possible intermediates in the conversion of D-glucuronic acid to ascorbic acid, a survey of a variety of pertinent hexoses and hexose de-

natives as precursors was carried out. The data are summarized in Table V.

It is seen that D-glucose, D-galactose, glucose-1-phosphate, glucose-6-

TABLE V  
*Precursors of Ascorbic Acid*

The test conditions were exactly as in Table I. Precursors were added in the amounts shown, total volume, 2.5 ml.

Experiment No	Substrate	Amount, $\mu$ moles	"Total" ascorbic acid		
			Zero time, $\mu$ moles	2 hours, $\mu$ moles	Net, $\mu$ moles
1	D-Glucuronolactone	10.0	0.40	1.83	+1.43
	D-Glucuronic acid	10.0	0.40	1.97	+1.57
	D-Galacturonic "	10.0	0.40	1.21	+0.80
2	D-Glucuronolactone	10.0	0.33	1.36	+1.03
	L-Gulonolactone	10.0	0.31	1.46	+1.15
	L-Galactonolactone	10.0	0.33	1.13	+0.80
	L-Ascorbic acid	10.0	1.34	1.20	-0.14
3	None		0.32	0.34	+0.02
	D-Glucuronolactone	12.5	0.36	0.90	+0.54
	D-Galacturonic acid	12.5	0.39	0.71	+0.32
	D-Gulonolactone	12.5	0.37	0.33	-0.04
	L-Ascorbic acid	0.67	1.00	0.87	-0.13
4	D-Glucuronolactone	10.0			+0.35
	Glucose-1-phosphate	10.0			0.00
	Glucose-6-phosphate	10.0			0.00
	D-Gulonolactone	10.0			-0.03
	D-Galactonolactone	10.0			+0.01
5	D-Glucuronolactone	10.0	0.211	0.900	+0.69
	L-Gulonic acid	10.0	0.193	0.815	+0.62
	" "	10.0	0.160	0.770	+0.61
6*	" "	10.0	0.197	0.674	+0.48
	D-Gulonic acid	10.0	0.209	0.215	+0.01
	D-Glucuronolactone	10.0	0.210	0.377	+0.17
7	"	10.0	0.27	0.72	+0.45
	L-Gulonic acid	10.0	0.27	0.71	+0.44
	L-Gulonolactone	10.0	0.27	0.52	+0.25

\* Mouse liver extract was used in this experiment.

phosphate, and uridine diphosphoglucose were inactive as precursors of ascorbic acid in the liver extract, although all these substances are probably capable of acting as precursors of glucuronic acid in the intact animal (14-16). It appears probable that the necessary preparatory enzymes either are not present or are inactive in the rat liver extracts under the conditions employed.

D-Glucuronic acid and D-glucuronolactone were found to be equally effective precursors under the conditions tested. D-Galacturonic acid was about 60 per cent as effective as D-glucuronic acid, pure D-galacturonolactone was not available for test.

Of greatest significance, however, is the fact that the liver extracts convert L-gulonic acid or its lactone to ascorbic acid at rates sufficiently high to suggest that it may be an intermediate in the conversion of D-glucuronic acid to L-ascorbic acid, according to the general mechanism postulated by Isherwood *et al* (5). Actually, in mouse liver extracts, L-gulonate was found to be a far more effective precursor of ascorbic acid than D-glucuno-

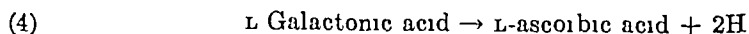
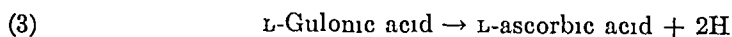
TABLE VI  
*Fractionation of Rat Liver Extract*

The test system was exactly as in Table I, with either D-glucuronolactone, 0.004 M, as substrate to test synthesis, or ascorbic acid, 0.0004 M, to test the rate of destruction. Where indicated, heated preparations had been placed in a boiling water bath for 5 minutes prior to test. Other details are given in the text.

Experiment No	Enzyme preparation	"Total" ascorbic acid, $\mu$ moles	
		Formed	Destroyed
1	Whole extract	0.53	0.47
	" " (heated)	-0.02	0.21
	Supernatant fluid (100,000 $\times$ g)	-0.02	0.18
	Residue	0.00	0.23
	Supernatant fluid + residue	0.73	0.53
2	Whole extract	1.28	0.58
	Heated residue + unheated supernatant fluid	-0.07	0.65
	Unheated residue + heated supernatant fluid	-0.03	0.31
	" " + unheated supernatant	1.26	0.47

lactone. Similarly, L-galactonolactone was also found to yield ascorbic acid, although at a lower rate than L-gulonic acid. In full support of these considerations is the finding that D-gulonic acid and its lactone were completely inactive as precursors of ascorbic acid.

From these results it may be concluded that Reactions 3 and 4 occur in the liver extracts, and in all likelihood represent a stage in the conversion of D-glucuronic acid or D-galacturonic acid to L-ascorbic acid.



*Fractionation of Liver Extract*—The rat liver extract used in these experiments may be separated into two heat-labile fractions, neither of which has any activity alone in formation of ascorbic acid from D-glucuronic acid. Upon recombination of these fractions, full activity is restored (Table VI).

The separation is brought about simply by centrifuging the rat liver extract at  $100,000 \times g$  for 30 minutes, a procedure which sediments microsomes and other small particulate elements. Furthermore, it was found that the sedimented fraction limits the rate of the synthesis of ascorbic acid, since the activity of a given amount of the supernatant fraction could be increased beyond the activity of the unfractionated extract by addition of more than an equivalent amount of the sedimented fraction. The experi-

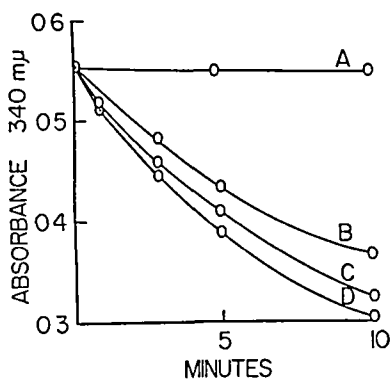


FIG 2

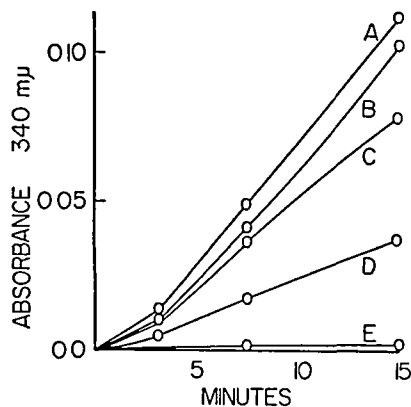


FIG 3

FIG 2 Oxidation of TPNH by D-uronic acids. The test system consisted of 0.05 M Tris buffer, pH 7.6, 0.11 M KCl, 0.01 M nicotinamide, TPNH, substrate, and 170  $\gamma$  of dialyzed protein fraction, precipitated between 33 and 50 per cent saturation with ammonium sulfate from the supernatant fluid remaining after centrifugation of rat liver extract at  $100,000 \times g$ . Total volume, 2.5 ml. Curve A indicates a change of absorbance in the absence of substrate, Curve B, D-glucuronolactone, 0.002 M, Curve C, D-glucuronate, 0.004 M, Curve D, D-galacturonate, 0.005 M. Temperature, 24°.

FIG 3 Reduction of TPN<sup>+</sup> and DPN<sup>+</sup> by L-aldehydic acids. The experimental details were as in Fig 2, pH 8.6, temperature, 24°. Curve A shows the rate of reduction of TPN<sup>+</sup> in the presence of L-gulonolactone, Curve B, reduction of TPN<sup>+</sup> by L-gulonate, Curve C, reduction of TPN<sup>+</sup> by L-galactonate, Curve D, reduction of DPN<sup>+</sup> by L-gulonate, Curve E, no substrate addition.

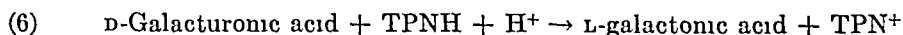
ments outlined in Table VI also show that part of the ascorbic acid destruction in the crude extract is non-enzymatic in nature.

The ability to separate two gross components of the enzyme system has simplified considerably the approach to individual enzymes involved in the biosynthesis.

*Oxidation of TPNH by D-Glucuronolactone*—The ability to separate the liver extract into two fractions suggested that each fraction might catalyze a separate stage in the conversion of D-glucuronolactone to L-ascorbic acid. It was found in fact that a dialyzed protein preparation obtained by ammonium sulfate fractionation of the supernatant fluid which remained after centrifugation of the liver extract at  $100,000 \times g$  catalyzed the oxidation of TPNH by D-glucuronolactone. Fig 2 shows that TPNH added alone to

the clear fraction is not oxidized, since there was essentially no change in absorbance at 340 m $\mu$ . However, when either D-glucuronic acid, D-glucuronolactone, or D-galacturonic acid was added, there was a decline in absorbance corresponding to oxidation of the TPNH. The rate of oxidation of TPNH by D-glucuronolactone was not significantly different from the rate with the free acid. The reduction products have not yet been identified with certainty. It appears likely that L-gulonic acid (or its lactone) is formed from D-glucuronic acid and L-galactonic acid (or lactone) from D-galacturonic acid by reversible, pyridine nucleotide-linked reactions, since, when either L-gulonate or L-galactonate was incubated with TPN<sup>+</sup> and the enzyme fraction at pH 8.6, reduction of TPN<sup>+</sup> was found to take place (Fig. 3). The rate and extent of reduction were consistent with the view that the conversion of the D-uronic acid to L-aldonic acid is reversible. Both the lactone and free acid forms of L-gulonic were active in the reverse direction, D-gulonic acid, the other stereoisomer, was not active. It was found that DPNH and DPN<sup>+</sup> could replace TPNH and TPN<sup>+</sup>, respectively, in the forward and reverse reactions, however, the rate of reaction with DPN<sup>+</sup> was considerably lower (Fig. 3). The rat liver fraction used in these spectrophotometric experiments was unable to convert either of the L-aldonic acids to L-ascorbic acid.

From these experiments it may be tentatively concluded that the rat liver fraction catalyzes the reversible Reactions 5 and 6



*Lactonase Activity of Rat Liver Extracts*—Because of the apparent dependence of the pertinent plant enzymes on lactone or ester forms of the uronic and aldonic acid precursors of ascorbic acid and because, on the other hand, the free acids as well as the lactones appear to be fully active in the rat liver extract here studied, the activity of the rat liver extract in catalyzing hydrolysis of the lactones of pertinent aldonic and uronic acids has been examined. Some typical findings (Fig. 4) show that the rat liver extract vigorously hydrolyzes the  $\gamma$ -lactones of D-glucuronic acid, L-gulonic acid, and L-galactonic acid. The course of the reactions was easily followed by measuring manometrically the liberation of acid groups in a CO<sub>2</sub>-bicarbonate buffer system. Some spontaneous, non-enzymatic, hydrolysis took place.

It is clear from the reaction curves shown that the aldonic and uronic lactones are hydrolyzed at rates considerably greater than the rate of formation of ascorbic acid in these extracts. However, over the 2 hour incubation periods in the assay for ascorbic acid synthesis, it would appear that significant concentrations of the lactones were usually present for a considerable

period following their addition to the test system as precursors, in addition to the free acids formed by their enzymatic hydrolysis. The samples of D-glucuronic acid and L-gulonic acid used were found to contain at the most only small amounts of the lactones, as determined by the hydroxamic acid test. If the enzymes concerned in ascorbic acid formation from D-glucuronic acid require the intermediates in the form of lactones or esters, then it is necessary to assume that the liver extracts employed in this study can form the lactones or esters from the free uronic and aldonic acids which are experimentally added as precursors.

The protein fractions described above, which catalyze the oxidation of

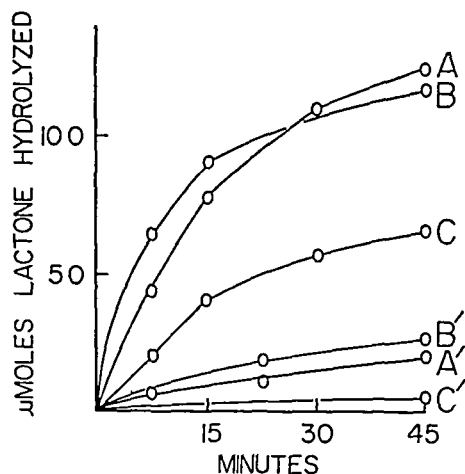


FIG 4 Enzymatic hydrolysis of lactones. The test system contained 0.02 M  $\text{NaHCO}_3$ , 0.02 M lactone, and 0.7 ml of rat liver extract or  $\text{H}_2\text{O}$  in a total volume of 2.5 ml. The reactions took place in Warburg vessels at  $37^\circ$ , gas phase, 95 per cent  $\text{N}_2$ -5 per cent  $\text{CO}_2$ . Curves A and A' represent the rates of enzymatic and non-enzymatic hydrolysis of D-glucuronolactone, Curves B and B', L-gulonolactone, and C and C', L-galactonolactone.

TPNH by D-glucuronic acid and its lactone, were also found to contain considerable lactonase activity. The lactonase activity of rat liver extracts has quite different specificity from the bacterial lactonase studied by Brodie and Lipmann (17).

*Other Properties of Enzyme System*—The rat liver extracts were usually utilized immediately after preparation. However, when kept frozen at  $-15^\circ$ , they retained essentially full activity in the conversion of D-glucuronolactone to ascorbic acid for at least 3 weeks. Extracts of acetone-dried rat liver had no activity. The optimal pH for the over-all reaction was found to be between 7.0 and 7.6. There was no activity at pH 6.0, at pH 8.0, 75 per cent of the maximal activity was observed.

The effect of some inhibitors was tested. Although  $5 \times 10^{-4}$  M cyanide

did not inhibit the synthesis, a concentration of 0.01 M reduced activity by one-half. Sodium azide at 0.008 M had no inhibitory action. It would therefore appear that cytochrome oxidase is probably not involved in the biosynthesis of ascorbic acid, the inhibition of the system at higher concentrations of cyanide could conceivably be caused by cyanohydrin formation with one or another of the reaction components. The synthesis is inhibited about 60 per cent by 0.008 M oxidized glutathione, about 60 per cent by 0.008 M iodoacetamide, and about 50 per cent by 0.008 M *p*-chloromercuribenzoate. About 60 per cent inhibition was caused by  $2 \times 10^{-4}$  M 2,4-dinitrophenol. Fluoride (0.008 M) and arsenate (0.008 M) inhibited only slightly.

Chloretone, which increases ascorbic acid excretion in rats, did not accelerate the activity of the liver enzyme system either when added *in vitro* or when administered to the rats prior to the preparation of the enzyme extract.

Attempts were made to improve the yield of ascorbic acid in these tests by addition of reduced glutathione, which is able to reduce dehydroascorbic acid non-enzymatically. However, although reduced glutathione reduced the rate of destruction of added ascorbic acid, it did not increase the rate of formation of ascorbate from glucuronic acid, concentrations above  $8 \times 10^{-3}$  M were significantly inhibitory.

*Distribution of Enzyme System in Different Species*—Preliminary experiments have revealed that the conversion of D-glucuronic acid to L-ascorbic acid occurs in extracts of the livers of the rat, mouse, rabbit, and dog, but not in liver extracts from the guinea pig, a species requiring dietary ascorbic acid. The liver extracts were prepared and the tests performed exactly as described for the case of rat liver.

A number of experiments were carried out in order to account for the failure of guinea pig liver extracts to form ascorbic acid. It was found that the liver extract of guinea pig destroyed added L-ascorbic acid at about the same rate as rat liver extract (Table VII), it thus appeared unlikely that an extraordinarily high rate of destruction in the guinea pig liver could be entirely responsible for the failure to detect formation of ascorbic acid. It was found that addition of the inactive guinea pig liver extract to rat liver extract of known activity caused some inhibition (35 per cent) of ascorbic acid formation by the latter (Table VII). However, the specificity of this inhibition can be questioned, since it was found that formation of ascorbic acid by rat liver extract was not proportional to concentration of the latter but actually fell off with increasing concentration beyond a certain point. Such effects could conceivably be caused by increased rates of enzymatic destruction of one or another cofactor, necessary for formation of ascorbic acid as the concentration of the extract in the test system is in-

creased. If the guinea pig liver extracts contain a specific inhibitory factor, it does not appear to be a potent one.

The most significant experiments were provided by tests of the supernatant fraction and the residue obtained by the centrifugation of the guinea pig liver extract at  $100,000 \times g$ . It will be recalled that the rat liver extract can be separated into two heat-labile fractions in this manner, both of which

TABLE VII  
*Enzymatic Defect in Liver Fractions of Guinea Pig*

Test system exactly as described in Table I. Rat liver and guinea pig liver extracts were prepared as described in the text. Each was then subjected to centrifugation at  $100,000 \times g$ , yielding a supernatant fraction and a residue fraction. These were tested singly or in combination, as shown below. Substrates were 0.004 M D-glucuronolactone or 0.0004 M L-ascorbic acid, as shown. Guinea pig = g p.

Experiment No	Enzyme preparation	Substrate	Net change in "total" ascorbic acid, $\mu$ moles
1	Rat liver extract	D-Glucuronolactone	+1.03
	G p " "	"	-0.05
2	Rat " "	L-Ascorbate	-0.45
	G p " "	"	-0.45
3	Rat " "	D-Glucuronolactone	+1.17
	G p " "	"	-0.04
	Rat " " + g p liver extract	"	+0.75
4	G p supernatant fraction	"	-0.04
	" residue fraction	"	-0.02
	" supernatant fraction + g p residue fraction	"	+0.01
	Rat supernatant fraction + rat residue fraction	"	+0.89
	G p supernatant fraction + rat residue fraction	"	+0.55
	G p residue fraction + rat supernatant fraction	"	+0.01

are required for conversion of D-glucuronic acid to L-ascorbic acid. The guinea pig liver extract, or the two subfractions derived from it, is inactive (Table VII), but, when the supernatant fraction of guinea pig liver are combined with the residue fraction of rat liver, the combination was found to be capable of forming ascorbic acid. Conversely, when the residue fraction of guinea pig was combined with the supernatant fraction of rat liver, no activity was obtained.

From these experiments it may be concluded tentatively that the inactivity of guinea pig liver extracts is probably not caused by the occurrence



of an extremely high rate of destruction of ascorbic acid or by the presence in the guinea pig of specific factors highly inhibitory to the enzymatic conversion of D-glucuronolactone to ascorbic acid. It appears more likely that a necessary enzyme or cofactor is lacking in the guinea pig liver extract, a factor which normally is present in rat liver extract in the fraction sedimenting at  $100,000 \times g$ .

#### DISCUSSION

The gross reaction pattern involved in the enzymatic conversion of D-glucuronic acid to ascorbic acid as it occurs in the liver extracts described here appears to be entirely consistent with the results of the isotopic investigations of King and his colleagues on the intact rat (1-4) and the reaction pattern postulated by Isherwood *et al* (5). There are obvious points of similarity between the enzymatic properties of the rat liver extracts and the plant extracts and plant mitochondria studied by the Cambridge group (6, 7). In both cases a D-uronic acid, or its lactone, is converted, presumably to the corresponding L-almonic acid, or lactone, in a pyridine-linked reduction step, and the L-almonic acid is then oxidized to L-ascorbic acid.

On the other hand, there appear to be some significant differences between the rat liver and the plant systems which deserve mention. The plant extracts and mitochondria appear to involve only D-galacturonolactone as precursor, with L-galactonolactone as intermediate (6, 7), whereas the rat liver extract is capable of utilizing either D-glucuronic or D-galacturonic acid as precursor, the former being more effective.

A second point of difference is that the plant extracts and mitochondria require the lactones or esterified forms of D-galacturonic acid and L-galactonic acid and do not act on the free acids (6, 7), whereas the rat liver extracts appear to be almost equally active with either the free acid or lactone forms of the D-uronic and L-almonic acid intermediates. Furthermore, the liver extracts catalyze hydrolysis of the lactones of the uronic and almonic acids at very high rates. The possible occurrence of enzymatic lactonization reactions in the rat liver extracts is not excluded, and is being investigated further. Obviously, in the conversion of free L-gulonic acid to L-ascorbic acid, a lactonization step must occur, although this need not necessarily be enzyme-catalyzed.

Another point of difference between the rat liver system and the plant system lies in the intracellular localization of the enzymes concerned. Mapson *et al* have found that mitochondria from germinating pea seedlings catalyze the conversion of L-galactonolactone to ascorbic acid (6), however, we have found that the mitochondria of rat liver have virtually no activity in any phase of the conversion of D-glucuronic or D-galacturonic acid to ascorbic acid. Essentially all the activity in these processes has been found to be in the soluble fraction and the microsomes of rat liver homogenates.

Furthermore, the formation of ascorbic acid in the liver extracts was not inhibited by low concentrations of cyanide, whereas the reaction in plant mitochondria was evidently dependent on cytochrome oxidase (6)

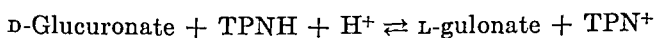
The requirement for ATP in the rat liver system suggests that enzymatic phosphorylation of an intermediate may occur. On the other hand, the ATP may be concerned in a secondary reaction, such as phosphorylation of DPN to form TPN. Uridine diphosphoglucuronic acid is known to be active in formation of glucuronides (14, 15), but all attempts to establish participation of uridine nucleotides in ascorbic acid formation from D-glucuronic acid yielded negative results. In fact, UTP was found to inhibit acid formation from D-glucuronic acid, relief of this inhibition was provided by glucose. It is premature, however, to eliminate the uridine nucleotides from further consideration in this connection. Obviously, further investigation on more purified enzyme preparations is required to establish the nature and the role of cofactors.

The two major reaction steps have been under more intensive investigation and will be reported on in more detail elsewhere. It is proposed to utilize this information directly in order to establish more precisely than has yet been possible (Table VII) the nature of the presumably gene-linked enzymatic defects in the tissues of primates and the guinea pig, which may explain the dependence of these species on dietary ascorbic acid.

#### SUMMARY

Rat liver extracts convert D-glucuronic acid or D-galacturonic acid or their lactones to ascorbic acid. After dialysis of the extracts, it was necessary to add adenosine triphosphate, diphosphopyridine nucleotide (DPN),  $Mg^{++}$ , and nicotinamide for full activity. Aging of the extracts revealed a requirement for triphosphopyridine nucleotide (TPN), a number of other coenzymes, including uridine nucleotides, was found to have no stimulating effect on the conversion. The liver extracts also destroyed added L-ascorbic acid at a high rate.

Both L-gulonic acid and L-galactonic acid, and their lactones, were converted to ascorbic acid at rates suggesting that they could be intermediates in formation of ascorbic acid from D-glucuronic acid and D-galacturonic acid, respectively, according to the mechanism suggested by Isherwood, Chen, and Mapson. The D-aldoic acids were not active as precursors. Fractionation of the liver extracts yielded a protein preparation capable of catalyzing the oxidation of TPNH by D-glucuronic acid and the reduction of  $TPN^+$  by L-gulonic acid, suggesting that Reaction 5



represents the first stage in the conversion of D-glucuronic acid to L-ascorbic acid. DPN could replace TPN in this reaction, but was much less active.

Both the free acid and lactone forms of the precursors were fully active in both stages of the conversion in liver extracts. The extracts contained a lactonase capable of hydrolyzing the lactones of the pertinent uronic and aldonic acids at very high rates.

The enzymatic formation of ascorbic acid from D-glucuronolactone was observed to occur in liver extracts of rat, mouse, dog, and rabbit, but not from the guinea pig, a species requiring dietary ascorbic acid. The failure of the guinea pig liver extracts in this conversion is probably not caused by the presence of an inhibitory factor or an excessively high rate of destruction of ascorbic acid, but rather by the absence of a heat-labile factor, which in rat liver, at least, is normally present in a protein fraction sedimented from the extract at  $100,000 \times g$ .

Differences in the plant and animal enzyme systems were discussed

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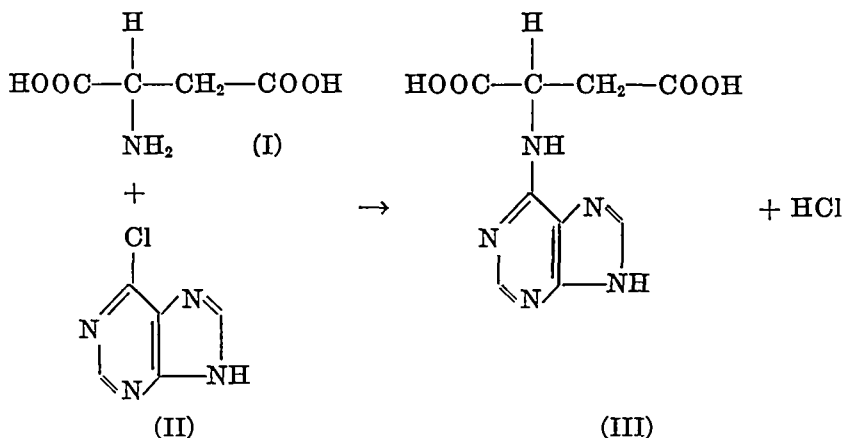
# SYNTHESIS OF 6-SUCCINOAMINOPURINE\*

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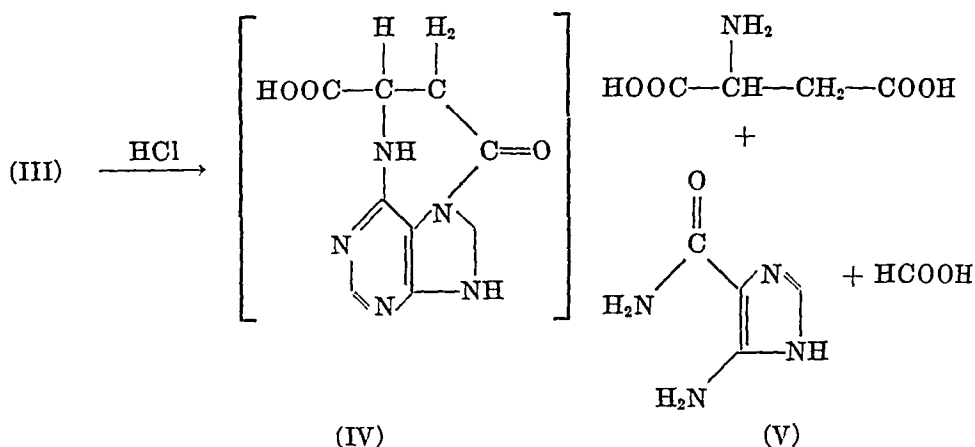
The structure proposed for adenylosuccinic acid (6-succinoaminopurine-9-ribosyl-5'-phosphate), the product of the enzymatic reaction of adenosine-5'-phosphate with fumaric acid (1, 2), is supported by a synthesis of the aglycone (III) from 6-chloropurine (II) and aspartic acid (I) reported in



this paper This reaction, which appears applicable to the condensation of several amino acids with 6-chloropurine, is conducted in aqueous solution at pH 9.5. The compounds formed exhibit an ultraviolet absorption maximum at 270 to 275 mμ in acid and those bearing two carboxyl substituents (derived from aspartic acid or glutamic acid) or a sulfonic and a carboxyl group (cysteic acid) are characterized by lability of the purine ring to acid hydrolysis with resultant formation of a diazotizable amine. The amine exhibits an ultraviolet absorption maximum at 267 mμ in acid, and is chromatographically identical with 4-amino-5-imidazolecarboxamide (V) and different from 4-amino-5-imidazolecarboxylic acid and 4-amino-imidazole, according to the chromatographic and spectral data for these compounds reported by Rabinowitz (3). In the case of the acid hydrolysis of 6-succinoaminopurine, which has been most extensively studied, aspartic acid is formed in addition to the diazotizable amine. It is suggested that the acid hydrolysis of 6-succinoaminopurine involves the formation of an

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intermediate anhydride (IV) by the internal condensation of a substituent carboxyl with the nitrogen of the imidazole ring corresponding to N-7 of



the purine. The breakdown of such an intermediate would account for the acid lability of 6-succinoaminopurine. However, chromatographic or spectrophotometric evidence has not been obtained in support of the existence of such an intermediate.

The identity of the natural aglycone, produced by mild acid hydrolysis of adenylosuccinic acid and 6-succinoaminopurine of synthetic origin, is based upon corresponding chromatographic behavior of the compounds in several solvent systems and with the anion exchange resin, on similar rates of degradation in acid with resultant formation of the same diazotizable amine, and on identical characteristics of spectrophotometric titration.

#### EXPERIMENTAL

*Synthesis of 6-Succinoaminopurine*—Preliminary investigation of the reaction of 6-chloropurine and aspartic acid in aqueous solution indicated that conditions were optimal at pH 9.5, and that both yield and ease of isolation of product were superior to those employing non-aqueous solvents with basic catalysis.

2 gm of 6-chloropurine (Dougherty Chemicals) and 1.2 gm of L-aspartic acid were added to 35 ml of water, and the mixture was brought to pH 9.5 with concentrated KOH. The solution was refluxed for 3 hours, cooled, and added to 1 liter of absolute ethanol. After standing overnight at 4°, the precipitate was separated by filtration and washed with 100 ml of cold absolute ethanol. After the precipitate was dried *in vacuo*, 2.15 gm of crude product were obtained, this exhibited an absorption maximum at 276 mμ in acid. From a predicted extinction coefficient at this wavelength for 6-succinoaminopurine of  $17.2 \times 10^3$ , based on a quantitative conversion of the parent nucleotide to the aglycone by acid hydrolysis (1)

and the presence of aspartic acid as determined by the ninhydrin reaction, the crude product was estimated to contain about 60 per cent 6-succinoaminopurine. Neither unchanged 6-chloropurine nor hypoxanthine could be detected in the crude product by paper chromatography.

Purification of 6-succinoaminopurine was achieved by dissolving 400 mg of the crude product in 20 ml of water (pH 8.0) and allowing this solution to percolate through a column 12 × 200 mm composed of 2 parts charcoal and 1 part Celite. The effluent contained no ultraviolet-absorbing material, and over 90 per cent of the aspartic acid of the crude product was recovered in this fraction. The column was then washed with water and successive 10 ml fractions of effluent were collected. The first two fractions, which contained 20 mg of 6-succinoaminopurine and a small amount of aspartic acid, were discarded. The subsequent 100 ml of effluent contained no amino acid and all of the remaining ultraviolet-absorbing material. This fraction was concentrated to 4 ml and added to 300 ml of absolute ethanol. After standing overnight at 4°, the crystalline precipitate was removed by filtration, washed with absolute ethanol, and dried *in vacuo*. Purified 6-succinoaminopurine was obtained in a yield of 175 mg as the dipotassium 2H<sub>2</sub>O salt with an extinction coefficient of  $17.6 \times 10^3$  in acid at 276 mμ (Table I). Analysis<sup>1</sup> of the compound dried *in vacuo* at 25° indicates 2 molecules of water of crystallization. Calculated for C<sub>9</sub>H<sub>8</sub>O<sub>4</sub>N<sub>5</sub>K<sub>2</sub> · 2H<sub>2</sub>O, C 29.8, H 3.6, O 26.6, N 19.4, found, C 28.5, H 3.07, O 26.7, N 18.8. The compound progressively darkens above 255° and decomposes with evolution of gas at 298°.

The reaction of 6-chloropurine with amino acids has been applied to glutamic acid, cysteic acid, glycine, lysine, and histidine. Conditions similar to the reaction described for the synthesis of 6-succinoaminopurine were employed with 200 mg of 6-chloropurine and a relative excess of amino acid of 0.5 M dissolved in 3.5 ml of water at pH 9.5. After refluxing for 3 hours, the mixture was poured into excess absolute ethanol and the precipitate collected by filtration. For the amino acids listed above, the ethanol precipitate contained crude substituted aminopurine, estimated by spectrophotometry to be equivalent to about 25 to 35 per cent of the theoretical yield. The principal contaminant was in each case the unchanged amino acid. Paper chromatography in several solvent systems showed a single ultraviolet-absorbing component (Table I). Because of the small amounts of these compounds and their high solubility in aqueous and non-aqueous solvents, further purification could not be achieved by crystallization, and the data presented for these compounds are intended to demonstrate the potential scope of the reaction rather than the preparation of definitive compounds. Each of the compounds shows an absorption maximum in the

<sup>1</sup> Huffman Microanalytical Laboratories

275  $m\mu$  region, and, by comparison with the natural and synthetic aglycone, is assumed to have an extinction coefficient at the maximum of approximately  $16$  to  $17 \times 10^3$ . On this basis, the crude products for which data are presented in Table I are approximately 50 to 60 per cent pure.

*Comparison of 6-Succinoaminopurine with Aglycone Derived from Adenyl-*

TABLE I  
*Paper Chromatography*

Compound	Absorbance $\times 10^3$ in 0.1 N HCl	$R_F$ paper chromatography			$R_F$ amine product of acid degradation		Ultraviolet absorption, $m\mu$			
		Solvent 1	Solvent 2	Solvent 3	Solvent 1	Solvent 2	0.1 N HCl		0.1 N NaOH	
	$\mu l$ , 276 $m\mu$						Maxi mum	Mini mum	Maxi mum	Mini mum
6-Succinoamino- purine	17.6	0.26	0.53	0.37	0.54	0.38	276	235	275	244
Aglycone from adenylosuccinate	17.2	0.26	0.53	0.37	0.54	0.38	276	235	275	244
6-Glutamyl purine	10.9	0.27	0.67	0.46	0.54	0.38	276	236		
6-Glycyl purine	12.5	0.40	0.61	0.46			275	235		
6-Cysteic acid purine	10.9	0.26	0.33	0.31	0.54	0.38	276	235		
6-Histidyl purine	8.9	0.42	0.57	0.46			278	236		
6-Lysyl purine	9.5	0.45	0.56	0.53			273	234		
6-Chloropurine	8.4	0.69	0.76	0.85			264			
Hypoxanthine		0.41		0.66						
4-Amino-5-imidazolecarboxamide		0.54	0.38		0.54	0.38	267			

Solvent 1: Concentration,  $\text{NH}_4\text{OH}$ , 10,  $\text{H}_2\text{O}$ , 20, isopropyl alcohol, 70. Double zoning in this solvent may be encountered, depending on the amount of inorganic cation present. Solvent 2, butanol, 50,  $\text{H}_2\text{O}$ , 25, acetic acid, 25. The 4-amino-5-imidazolecarboxamide standard was put on the paper in 1 N HCl solution. Solvent 3, 1 M  $\text{NH}_4\text{Ac}$ , 3.5, ethanol, 7.0.

*osuccinic Acid by Acid Hydrolysis*—The aglycone of adenylosuccinic acid has not been recovered as a solid, following acid hydrolysis of the parent nucleotide (1, 2), because of the limited amounts of the nucleotide available and the high solubility of the aglycone and its salts in a variety of solvents. The identification of the aglycone with 6-succinoaminopurine is based on chromatographic and spectrophotometric criteria and the characteristic lability of the compound in acid to yield a diazotizable amine. Table I

summarizes the ultraviolet absorption characteristics and the chromatographic behavior of the natural and synthetic aglycone and the diazotizable amine derived by acid hydrolysis

These compounds may also be isolated and determined by anion exchange chromatography. On a Dowex 1, 2 per cent cross-linked, 200 to 400 mesh resin column in the acetate form, 10 cm long and with a 1 cm diameter, the products of a 10 hour hydrolysis of adenylosuccinic acid (10  $\mu$ moles) and an equivalent amount of the synthetic aglycone in 0.5 N HCl at 100° were compared. Under these conditions some of the aglycone and the derived diazotizable amine remained at the end of the hydrolysis period. In both cases the diazotizable amine with an ultraviolet absorption maximum at 267  $m\mu$  was eluted from the column with 0.05 M  $\text{NH}_4\text{Ac}$  adjusted to pH 4.0. Treatment of the column with 0.25 M  $\text{NH}_4\text{Ac}$ , pH 4.0, removed 6-succinoaminopurine and the aglycone derived from adenylosuccinic acid in a single symmetrical peak between 60 and 120 ml of effluent.

Spectrophotometric characteristics dependent upon the dissociating groups of the natural and synthetic aglycone of adenylosuccinic acid are shown in Fig. 1. The data demonstrate the correspondence of the natural and synthetic aglycone and suggest the presence of two groups with pK values of 4.0 and 5.3. These dissociations are assigned to the substituted amino and the secondary carboxyl group, though not necessarily respectively. In contrast to the nucleotide, in which a dissociation at pH 2.1 attributed to a carboxyl group clearly influences spectrophotometric ratios (2), no corresponding spectrophotometric evidence for this dissociation is seen in the data for the aglycone presented in Fig. 2. However, electrometric titration with HCl of the purified synthetic aglycone, 6-succinoaminopurine, resulted in the consumption of 2.92 equivalents of acid per mole of 6-succinoaminopurine between pH 8.0 and 2.0 and indicates the presence of two free carboxyl groups and the substituted amino group.

The lability of the purine ring of 6-succinoaminopurine and the corresponding glutamic acid and cysteic acid derivatives to acid hydrolysis characterizes this group of compounds and affords another basis of correspondence between the natural and synthetic aglycones. In Fig. 2 the production of diazotizable amine by acid hydrolysis from a series of related compounds is shown. In the case of the aglycone of adenylosuccinic acid, synthetic 6-succinoaminopurine, and the analogous glutamic acid and cysteic acid derivatives, acid hydrolysis is associated with a decreasing absorption at 265 and 275  $m\mu$ , a decrease in the ratio of absorption of 265  $m\mu$  to 275  $m\mu$ , and the appearance and destruction of diazotizable amine. These data for 6-succinoaminopurine are recorded in Table II. Paper and ion exchange chromatography of hydrolysis mixtures of these compounds show the progressive disappearance of the substituted purine with the



production of a common ultraviolet-absorbing component which exhibits a maximum at  $265\text{ m}\mu$  in acid and reacts with the Bratton-Marshall reagent. The diazotizable amine accounts for about 40 per cent of the original purine, and is chromatographically identical with 4-amino-5-imidazolecarboxamide (Table I). The amine product of acid degradation of the

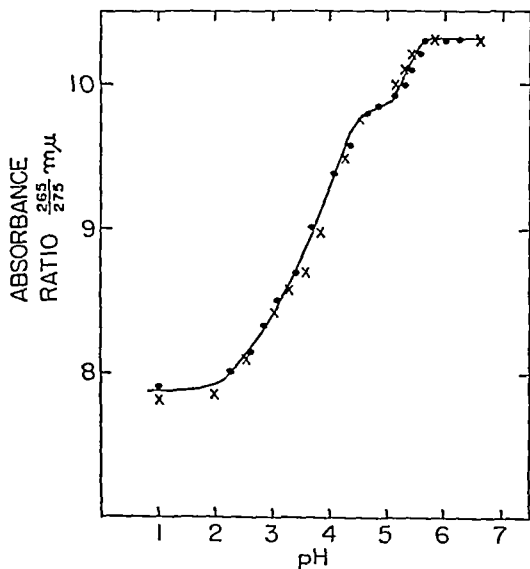


FIG 1

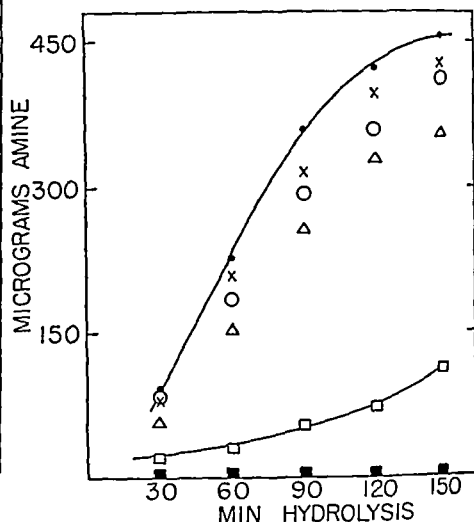


FIG 2

Fig 1 The natural aglycone was derived from adenylosuccinic acid by hydrolysis of the nucleotide in 1 N HCl for 15 minutes at  $100^\circ$ , conditions which have been shown to lead to complete hydrolysis of the riboside linkage without degradation of the purine moiety (1, 2). An aliquot of the hydrolysis mixture (●, natural) was added to 0.1 N HCl in amount sufficient to give an absorbance (density) reading at  $276\text{ m}\mu$  of 0.800. Purified synthetic 6-succinoaminopurine (×, synthetic) was dissolved in 0.1 N HCl at a comparable concentration, and the solutions were titrated with 2 N KOH at the glass electrode.

Fig 2 10  $\mu$ moles of each compound, determined spectrophotometrically, were dissolved in 2 ml of 1 N HCl and heated in a boiling water bath. Samples of 0.05 ml were withdrawn at intervals and diazotizable amine was determined by the Bratton-Marshall procedure. Adenylosuccinic acid, ●, 6-succinoaminopurine, ×, 6-glutaroaminopurine, ○, cysteic acid analogue, △, glycine analogue, □, hypoxanthine, ■.

substituted purines has not been further characterized, but from the foregoing evidence and the spectral data for 4-amino-5-imidazolecarboxylic acid and 4-aminoimidazole, as well as the extreme lability of these compounds reported by Rabinowitz (3), the amine is believed to be 4-amino-5-imidazolecarboxamide.

While amino acid-substituted purines such as kinetin (6-furfurylamino purine) (4) and 6-methylaminopurine (5) are resistant in the purine ring

to mild acid degradation, Fig 2 shows that the glycine-substituted purine is to some extent degraded in 1 N acid to diazotizable amine. In the case of 6-chloropurine, small amounts of diazotizable amine are formed, but

TABLE II  
*Acid Hydrolysis of 6-Succinoaminopurine*

10  $\mu$ moles of the purified dipotassium salt of 6-succinoaminopurine were dissolved in 2 ml of 1 N HCl and heated in a stoppered tube in a boiling water bath. Samples of 0.05 ml were withdrawn at intervals for spectrophotometry, after dilution to 4 ml with H<sub>2</sub>O, and for diazotizable amine determination by the Bratton-Marshall method.

Time	Ratio of absorbance	Diazotizable amine	Time	Ratio of absorbance	Diazotizable amine
<i>min</i>	275 m $\mu$ /265 m $\mu$	$\mu$ moles	<i>min</i>	275 m $\mu$ /265 m $\mu$	$\mu$ moles
0	1.25	0	180	1.09	4.40
30	1.24	0.75	210		4.1
60		1.75	240	1.00	3.6
90	1.17	2.30	270	0.95	3.5
120		3.20	330	0.94	3.2
150	1.13	3.60			

TABLE III  
*Chromatography of Acid Degradation Products of Carboxyl-Labeled Adenylosuccinic Acid*

Carboxyl-labeled adenylosuccinic acid was added to the unlabeled compound to give a final concentration of 14 mg of adenylosuccinic acid in 4 ml of 1.0 N HCl containing a total radioactivity of 254,000 c.p.m. as determined for an infinitely thin layer in a windowless counter. The solution was refluxed for 5 hours, diluted to 100 ml, and adjusted to pH 3.0. This solution was then percolated through a column of 1 cm diameter and 10 cm long of Dowex 50-H<sup>+</sup>, 200 to 400 mesh.

Eluting agent	Compound	Radio activity	Ninhydrin test	Arylamine test	Ultraviolet maximum, m $\mu$
		<i>per cent</i>			
0.5 N HCl	Aspartic acid	74	+	0	0
1.0 " "	Succinoaminopurine	8	0	0	276
2.0 " "	4-Amino-5-imidazolecarboxamide	0	0	+	267

lability is to a great extent masked by the rapid transformation of this compound in acid solution to hypoxanthine, which is resistant to acid degradation.

The products of acid hydrolysis were further characterized by employing a sample of adenylosuccinic acid labeled with C<sup>14</sup> in the two carboxyl groups.

(1, 2) The hydrolysate was resolved on a cation exchange resin in the  $H^+$  form. The 0.5 N HCl eluate, which was shown by paper chromatography to contain only aspartic acid (Table III), accounted for most of the original radioactivity, and this was identified with aspartic acid. A small amount of activity was associated with the unhydrolyzed 6-succinoaminopurine, but the only other ultraviolet-absorbing component, the amino-imidazole product of hydrolysis, was devoid of radioactivity. In acid hydrolysates of 6-succinoaminopurine, which result from prolonged heating in 3 N HCl, small amounts of  $\beta$ -alanine arising from decarboxylation of aspartic acid, and glycine arising from degradation of the imidazole nucleus, have been detected by paper chromatography.

Alkaline hydrolysis destroys 6-succinoaminopurine without formation of diazotizable amine, but the products of this reaction have not been identified.

#### SUMMARY

A compound chromatographically and spectrophotometrically identified as the aglycone of adenylosuccinic acid was synthesized from 6-chloropurine and aspartic acid and assigned the structure, 6-succinoaminopurine. The reaction appears applicable to several amino acids.

Synthetic 6-succinoaminopurine and the natural compound derived from adenylosuccinic acid exhibited lability of the purine ring to 1 N acid hydrolysis at 100° and yielded 4-amino-5-imidazolecarboxamide and aspartic acid under these conditions.

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# UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY CADMIUM ION\*

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Phosphorylation coupled to the aerobic oxidation of succinate or reduced diphosphopyridine nucleotide is uncoupled by low concentrations of a variety of compounds of unrelated structure like nitrophenols (2, 3), gramicidin (3), and calcium ions (4). The present report describes uncoupling by cadmium ions at low concentrations and recoupling in the presence of EDTA<sup>1</sup> or dithiols.

## EXPERIMENTAL

Mitochondria from rat liver were isolated in 0.25 M sucrose according to Schneider (5) and suspended in 1 ml. of 0.25 M sucrose per gm. of fresh liver. Rabbit liver mitochondria, used only for the experiments in Table I, were prepared by grinding the tissue in 0.25 M sucrose in a Waring blender at reduced speed (6) and were suspended in 1 ml. of sucrose solution per 2 gm. of liver.

The reaction mixture (3 ml.) for measuring oxidation and coupled phosphorylation contained 0.013 M potassium phosphate at pH 7.4, 0.002 M ATP, 0.017 M glucose, 0.003 M magnesium chloride, 0.05 mg. of hexokinase, 0.007 M potassium fluoride, 0.017 M succinate, and mitochondrial suspension.

\* A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists at San Francisco, April, 1955 (1).

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<sup>1</sup> The following abbreviations will be used: ATP, adenosine triphosphate, DNP, 2,4-dinitrophenol, EDTA, ethylenediaminetetraacetic acid, BAL, 2,3-dimercaptopropanol (British Anti-Lewisite), P/O, ratio of micromoles of phosphate esterified to microatoms of oxygen consumed.

equivalent to 0.3 to 1.0 gm of liver. Sucrose was added to give a final concentration of 0.25 M. The center well of the reaction vessel contained 0.2 ml of 5 N KOH. Oxygen consumption was measured in the Warburg apparatus at 30° for 15 to 20 minutes in air. The flasks were equilibrated in the bath for 6 minutes. Presumably, the reaction proceeded at the same rate during this period as in the next 6 minutes. Additional components and deviations from these experimental conditions are stated in the text or in Tables I to VIII.

For protein determination, 0.05 to 0.1 ml of mitochondrial suspension

TABLE I  
*Inhibitor from Aged Mitochondrial Preparations of Rabbit Liver*

Experiment No	Further additions	Oxygen	P/O
		<i>microatoms</i>	
1	None	16.9	0.21
	EDTA ( $1 \times 10^{-3}$ M)	15.2	0.51
	" + S (1.0 ml)	18.9	0.06
2	" ( $1 \times 10^{-3}$ M)	17.5	0.47
	" + S (0.9 ml)	19.2	0.00
	" + heated S (0.9 ml)	18.9	0.00
3	" ( $1 \times 10^{-3}$ M)	16.9	1.30
	" + heated S (1.0 ml)	20.3	0.00

Experiment 1, the rabbit liver mitochondrial suspension was stored at 0° for 60 hours and then centrifuged. The supernatant fluid is referred to as S. The residue was washed twice with 0.25 M sucrose containing  $1 \times 10^{-3}$  M EDTA and resuspended in the original volume of sucrose. Experiment 2, as Experiment 1, except that mitochondria were aged for 48 hours. Experiment 3, freshly prepared mitochondria were used. The S in this experiment was derived from mitochondria that had been aged for 48 hours. The supernatant fluid, heated in a boiling water bath for 5 minutes, was used where indicated. In each case, 0.5 ml of the mitochondrial preparation or washed residue (approximately 10 mg of protein) was used.

was first mixed with 0.2 ml of 5 per cent deoxycholate, and, after 10 minutes, the biuret reagent (7) was added. The protein concentration was calculated from absorbency measurements with bovine serum albumin as the standard. Phosphate was measured according to Fiske and Subbarow (8), and the hexokinase preparation was carried to Step 3a of the procedure of Berger and coworkers (9).

BAL and propane-1,3-dithiol were purchased from the Aldrich Chemical Company, ATP was supplied by the Sigma Chemical Company, and radioactive cadmium nitrate ( $\text{Cd}^{115}$ ) was obtained from the Atomic Energy Commission.  $\text{Cd}^{115}$  has a half life of 43 days and is a  $\beta$  emitter (1.7 m.e.v.). The samples were plated on 1 inch aluminum plates and radioactivity was

measured in a Tracerlab, Geiger-Muller tube, counter. The experimental samples and the standards were counted under identical conditions in order to correct for decay, back-scattering, and self-absorption.

### Results

During the course of the experiments on restoration of phosphorylation in mitochondria that had been subjected to various types of mild treatments, it was observed that, on prolonged storage at 0°, an inhibitor was released from rabbit liver mitochondria into the sucrose medium (Table I). The residue, which was washed with sucrose-EDTA solution, showed re-

TABLE II  
*Relative Effects of Various Cations on Phosphorylation  
Coupled to Succinate Oxidation*

Salt	Concentration	Oxygen	P/O
	$M \times 10^6$	<i>microatoms</i>	
None		13.3	1.64
CdCl <sub>2</sub>	3.3	9.3	0.14
ZnCl <sub>2</sub>	3.3	9.8	1.30
Pb(NO <sub>3</sub> ) <sub>2</sub>	3.3	12.6	1.61
HgCl <sub>2</sub>	3.3	11.6	1.68
CuSO <sub>4</sub>	3.3	10.7	1.70
CdCl <sub>2</sub>	1.7	11.6	0.72
ZnCl <sub>2</sub>	1.7	11.2	1.80

The rat liver mitochondria (8.0 mg of protein) were mixed with the above salts in 1.0 ml of 0.25 M sucrose in Warburg flasks and allowed to stand for 10 minutes at 0°. The components listed in the text were then added. The concentrations of the salts above are in the final volume of 3.0 ml.

sidual phosphate uptake which was increased by further addition of EDTA to the incubation medium. When the supernatant fluid was also present, phosphorylation was uncoupled. The inhibitor in the supernatant fluid was not destroyed by heating and subsequent removal of the coagulated protein. More striking evidence for the presence of the inhibitor is seen in Experiment 3, in which phosphorylation in freshly prepared mitochondria was completely uncoupled by the supernatant fluid. The effect of supernatant fluid was independent of the effect of EDTA.

In order to test the possibility that the inhibition was caused by metal ions released during aging, *e.g.* copper from butyryl CoA dehydrogenase (10) and zinc from glutamic dehydrogenase (11), the experiments presented in Table II were carried out. It was found that Cd<sup>++</sup> uncoupled phosphorylation at extremely low concentrations, the ion next in potency

being  $\text{Zn}^{++}$ . Other metal ions inactive at  $3.3 \times 10^{-6}$  M concentration include  $\text{Ba}^{++}$ ,  $\text{Ca}^{++}$ ,  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ ,  $\text{Be}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Fe}^{+++}$ , and  $\text{Al}^{+++}$ . Similar experiments have been carried out by Hunter and Ford (12), except that

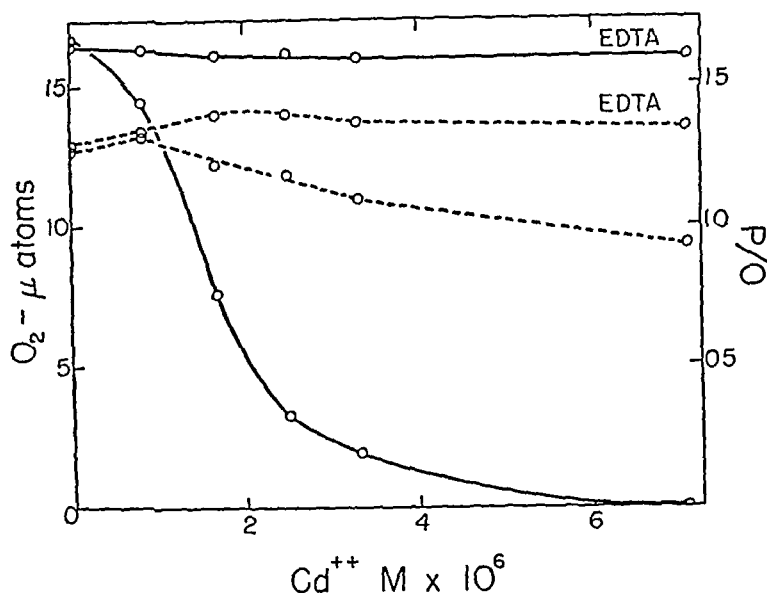


FIG 1 Uncoupling of phosphorylation by  $\text{Cd}^{++}$  and reversal by EDTA. Rat liver mitochondria (7.6 mg of protein) were incubated with cadmium chloride in 1.0 ml of 0.25 M sucrose at  $0^\circ$  for 10 minutes and then EDTA ( $3.0 \mu\text{moles}$ ) was added to one set of flasks. After another 5 minutes the remaining components were added. The  $\text{Cd}^{++}$  was added in amounts to give the above final concentration in 3.0 ml. The solid lines represent P/O and the dotted lines show oxygen taken up in 15 minutes.

TABLE III  
*Reversal of Uncoupling by Dithiols*

$\text{Cd}^{++}$	Reagent	Oxygen	P/O
		<i>microatoms</i>	
None		11.2	1.21
$3.3 \times 10^{-6}$ M		9.2	0.24
$3.3 \times 10^{-6}$ "	BAL ( $3.3 \times 10^{-6}$ M)	11.5	0.90
$3.3 \times 10^{-6}$ "	" ( $16.5 \times 10^{-6}$ M)	10.8	1.24
$3.3 \times 10^{-6}$ "	Propane-1,3-dithiol ( $3.3 \times 10^{-6}$ M)	11.6	0.95
$3.3 \times 10^{-6}$ "	" ( $16.5 \times 10^{-6}$ M)	10.2	1.24

the reaction mixture which they used contained tris(hydroxymethyl)-aminomethane buffer and higher concentrations of the metals.  $\text{Be}^{++}$  at  $3 \times 10^{-5}$  M had an effect similar to fluoride, it depressed respiration without changing the phosphate uptake so that a net increase in the value of P/O was obtained. Half maximal uncoupling of succinate oxidation occurred at  $1.6 \times 10^{-6}$  M  $\text{Cd}^{++}$  and complete uncoupling at  $5 \times 10^{-6}$  M (Fig

1) It is of interest that approximately 10 times this concentration of DNP is necessary to uncouple phosphorylation completely. Concentrations of  $\text{Cd}^{++}$  higher than  $10^{-5}$  M produced significant inhibition of the oxidative rate. Barron and Kalnitsky (13) have previously shown inhibition of succinate oxidation by  $\text{Cd}^{++}$  in a non-phosphorylating system. Hexokinase was not inhibited, even at  $5 \times 10^{-2}$  M  $\text{Cd}^{++}$ .

*Reversal of Uncoupling*—The uncoupling effect of  $\text{Cd}^{++}$  is completely overcome by  $10^{-3}$  M EDTA (Fig. 1). Dithiols are much more active reversing agents than EDTA. At a 1:1 concentration of BAL or 1,3-dithiopropane to  $\text{Cd}^{++}$ , the inhibition was reversed approximately 70 per cent (Table III). EDTA was virtually ineffective at these low levels. Glutathione did not reverse the uncoupling even at  $6 \times 10^{-3}$  M.

TABLE IV

*Effect of Cadmium Ion on Phosphorylation Coupled to Citrate Oxidation*

$\text{Cd}^{++}$	BAL, $1 \times 10^{-4}$ M	Oxygen <i>microatoms</i>	P/O
	—	9.5	2.61
	+	10.3	2.57
$0.67 \times 10^{-6}$ M	—	9.8	2.18
$0.67 \times 10^{-6}$ "	+	9.1	2.35
$1.7 \times 10^{-6}$ M	—	5.3	1.25
$1.7 \times 10^{-6}$ "	+	10.2	2.31
$5 \times 10^{-6}$ M	—	2.3	0
$5 \times 10^{-6}$ "	+	9.7	2.41

The mitochondria (from 0.5 gm. of rat liver) were mixed with  $\text{Cd}^{++}$  in 1.0 ml. of 0.25 M sucrose. After 10 minutes BAL was added to one set of flasks. The reaction components (see the text) were added 5 minutes after adding BAL.

Table IV shows that  $\text{Cd}^{++}$  also uncoupled phosphate esterification associated with the oxidation of citrate. The oxidation of citrate was more sensitive to  $\text{Cd}^{++}$  than was the oxidation of succinate.

$\text{Mn}^{++}$ ,  $\text{Co}^{++}$ , and  $\text{Ni}^{++}$  also counteract the effect of  $\text{Cd}^{++}$  on phosphorylation (Table V). The uncoupling of phosphorylation produced on incubation of mitochondria in phosphate buffer at  $30^\circ$  in the absence of substrate (14) is partially reversed by  $\text{Mn}^{++}$  but not by  $\text{Co}^{++}$  or  $\text{Ni}^{++}$ .<sup>2</sup> This difference in cation specificity is discussed later (see "Discussion"). At concentrations of  $10^{-3}$  M or higher,  $\text{Mn}^{++}$  inhibits phosphorylation (Table V).

#### *Binding of $\text{Cd}^{++}$*

$\text{Cd}^{++}$  added to mitochondria is bound strongly and is not removed appreciably by repeated washing with sucrose (Table VI). It may be noted

<sup>2</sup> Unpublished data.



that, after four washings, the P/O value was still low, but increased to 1.20 when BAL was added. In another experiment mitochondria were treated with  $\text{Cd}^{++}$  as described in Table VI, washed twice, and then dialyzed for 12 hours with vigorous stirring against 100 volumes of sucrose. The P/O value of the dialyzed preparation was 0.17 and rose to 1.62 on the addition

TABLE V  
*Restoration of Phosphorylation by  $\text{Mn}^{++}$ ,  $\text{Co}^{++}$ , and  $\text{Ni}^{++}$   
in Mitochondria Treated with  $\text{Cd}^{++}$*

Experiment No	Additions	Concentration	Oxygen	P/O
		<i>M</i>	<i>microatoms</i>	
1			11.0	0.34
	BAL	$1.7 \times 10^{-4}$	12.2	1.78
	$\text{MnCl}_2$	$3.3 \times 10^{-5}$	10.8	0.45
	"	$1.0 \times 10^{-4}$	13.6	1.42
	"	$1.7 \times 10^{-4}$	12.1	1.65
	"	$1 \times 10^{-3}$	10.8	0.78
2	$\text{NiCl}_2$	$1.7 \times 10^{-4}$	12.6	1.21
			16.0	0.36
	BAL	$1.7 \times 10^{-4}$	16.8	1.89
	$\text{MnCl}_2$	$1.7 \times 10^{-4}$	14.7	1.73
	$\text{CoCl}_2$	$3.3 \times 10^{-5}$	19.7	0.31
	"	$1.7 \times 10^{-4}$	19.2	0.80
3	"	$5 \times 10^{-4}$	15.1	1.18
			22.0	0.05
	BAL	$1.7 \times 10^{-4}$	22.0	1.28
	$\text{MnCl}_2$	$1.7 \times 10^{-4}$	23.4	1.06
	$\text{CoCl}_2$	$1.7 \times 10^{-4}$	19.6	0.35
	"	$5.0 \times 10^{-3}$	13.1	0.88

The mitochondrial preparation was mixed with an equal volume of  $5 \times 10^{-5} M$  cadmium chloride. After 10 minutes the suspension was centrifuged and the residue was washed once with sucrose. The  $\text{Cd}^{++}$ -treated enzyme (from 0.5 gm. of liver) was incubated with the compounds indicated above at  $0^\circ$  for 5 minutes before addition of the reaction medium. The final concentrations in 3.0 ml. are shown in the second column. Experiment 1 was carried out at  $30^\circ$  for 15 minutes, with Experiments 2 and 3 at  $37^\circ$  for 15 minutes.

of BAL. The control without  $\text{Cd}^{++}$  which was dialyzed similarly had a P/O of 1.78.

The strong binding of  $\text{Cd}^{++}$  suggested that radioactive  $\text{Cd}^{115}$  might be used as a marker to isolate the Cd-bound component from mitochondria. It was first confirmed that the bound radioactive  $\text{Cd}^{115}$  did not decrease measurably after four washings. Table VII shows that virtually all of the  $\text{Cd}^{115}$  added to mitochondria, even at a level 10 times that necessary for 100 per cent uncoupling, was bound. This continued binding of  $\text{Cd}^{++}$

beyond the point of complete uncoupling unfortunately introduced an uncertainty in the proposed isolation of the  $\text{Cd}^{115}$ -bound component of the

TABLE VI  
*Effect of Washing on Bound  $\text{Cd}^{++}$*

Treatment	No BAL		BAL ( $1.7 \times 10^{-4} \text{ M}$ )	
	Oxygen	P/O	Oxygen	P/O
	<i>microatoms</i>		<i>microatoms</i>	
None	23.5	0.25	20.3	1.89
1 washing	20.8	0.24	19.8	1.60
3    "	22.9	0.09	22.0	1.36
4    "	19.2	0.11	22.3	1.20

The mitochondria (10 ml) were treated with cadmium chloride, as shown in Table V, and washed each time with 50 ml of 0.25 M sucrose. The residue after each washing was made up to a volume corresponding to the original after allowing for withdrawal of sample for assay. Approximately 9 mg of protein were used in each flask. The rest of the conditions are as in Table V and in the text.

TABLE VII  
*Binding of  $\text{Cd}^{115}$  by Mitochondria*

Medium	Total $\text{Cd}^{115}$		
	Added	Protein bound	Supernatant
	<i>c p m</i>	<i>c p m</i>	<i>c p m</i>
Sucrose, 0.25 M	770	840	0
	1,160	1,090	54
	3,860	3,780	54
	11,600	12,000	72
Sucrose + reaction mixture	770	760	0
	1,160	1,040	0
	11,600	12,800	60
Sucrose + phosphate, 0.005 M	1,230	1,180	0
	3,900	3,620	0
Sucrose + Na bicarbonate, 0.005 M	1,230	1,280	
	3,900	4,100	

The mitochondria (1 ml containing 20.6 mg of protein) were mixed at  $0^\circ$  with  $\text{Cd}^{115}$  (24,600 c p m per  $\mu\text{mole}$ ) in 6.0 ml of the medium and centrifuged after 10 minutes. Aliquots of the well drained residue and supernatant fluid were plated for counting. 1000 c p m correspond approximately to level of  $\text{Cd}^{115}$  necessary to produce 100 per cent uncoupling.

phosphorylation system. Even a high ionic environment (1 M KCl) did not change the pattern of  $\text{Cd}^{115}$  binding at both low and high  $\text{Cd}^{++}$  concentrations.

The experiments described in Table VIII were carried out in order to study the mechanism of reversal of uncoupling by BAL or  $Mn^{++}$ . The wash liquid containing BAL extracted less than 3 per cent of the  $Cd^{115}$ , nevertheless, phosphorylation was fully restored. On the other hand, washing with  $MnCl_2$  did not restore phosphorylation unless BAL or  $MnCl_2$  was present during the subsequent measurement of P/O. This suggests that BAL and  $MnCl_2$  act by different mechanisms.

The bound  $Cd^{115}$  (mitochondria treated as in Table VIII) remained entirely on the coagulated protein when the suspension was heated in a boiling water bath for 3 minutes. Denaturation by 5 per cent trichloroacetic

TABLE VIII  
*Effect of Washing with BAL and Manganese Chloride  
on Bound  $Cd^{115}$  and Phosphorylation*

Washing medium	C p m per 21 mg of protein		P/O	
	Supernatant	Residue	Without BAL	With BAL
None		1830	0	1.41
Sucrose	6	1682	0	1.40
BAL	44	1720	1.61	1.48
$MnCl_2$	12	1710	0	1.52

The mitochondrial suspension (10 ml containing 210 mg of protein) was mixed with 0.8 ml of 0.001 M  $Cd^{115}$  (24,000 c p m per  $\mu$ mole). After 10 minutes the suspension was centrifuged and the residue was washed twice with 20 ml of sucrose and resuspended in 10 ml of sucrose. 1 ml of preparation was counted as such for the first line above. A 2.0 ml aliquot of suspension was diluted with 6 ml of sucrose and centrifuged after 10 minutes. The residue was resuspended in 2.0 ml volume (second line). The other washings were similar, except that the diluting sucrose (6 ml) contained BAL ( $1.7 \times 10^{-4}$  M) in one case and  $MnCl_2$  ( $1.7 \times 10^{-4}$  M) in the other. The initial supernatant fractions and the washed residues were counted. Phosphorylation coupled to succinate oxidation was measured with the washed residue as described in the text with and without addition of BAL.

acid, however, resulted in complete extraction of the  $Cd^{115}$ . When mitochondria containing bound  $Cd^{115}$  were exposed to distilled water for 10 minutes and then centrifuged at  $100,000 \times g$  for 30 minutes, approximately 25 per cent of the radioactivity appeared in the clear supernatant fluid. Further extraction of the residue with 0.1 M KCl yielded 20 per cent of the originally bound  $Cd^{115}$ . Under these conditions, although the mitochondrial structure is disrupted, the washed residue still retains the potential capacity for coupled phosphorylation (14).

#### DISCUSSION

The data presented above show that  $Cd^{++}$  is a powerful uncoupling agent and that its effect is reversible. The few preliminary experiments on the

mechanism of attachment of  $\text{Cd}^{++}$  have not been pursued in detail since apparently a considerable part of the  $\text{Cd}^{++}$  may be bound in a "non-specific" manner. Present knowledge of the system does not permit a distinction between the two types of binding.

Two possible schemes to account for the uncoupling by  $\text{Cd}^{++}$  have been examined in these experiments. First,  $\text{Cd}^{++}$  might displace another cation essential for phosphorylation from its active site.  $\text{Cd}^{++}$ -treated mitochondria can be washed extensively or dialyzed and still be reactivated by BAL. In order to support the above hypothesis, therefore, it is also necessary to assume that the displaced cation remains tightly bound at some other site and returns to the original active center when BAL is added to the system. The second hypothesis that could account for the uncoupling is that  $\text{Cd}^{++}$  might block a free active site. It is obvious from the data that a disulfide group on the enzyme could reversibly bind  $\text{Cd}^{++}$  at extremely low concentrations. Imidazole is also a possible but less likely candidate. Tanford (15) has found that  $\text{Cd}^{++}$  is bound by the imidazole groups of serum albumin. However, the strength of binding was greater with  $\text{Cu}^{++}$  and  $\text{Zn}^{++}$ , which is the reverse of the order of efficiency of uncoupling ( $\text{Cd}^{++}$ ,  $\text{Zn}^{++}$ ,  $\text{Cu}^{++}$ ).

When  $\text{Cd}^{115}$ -treated mitochondria are washed with BAL, negligible activity is extracted in the washings, although the enzymatic activity of the residue is fully restored (Table VIII). This unexpected observation is hard to explain. It is conceivable that the small amount of  $\text{Cd}^{115}$  recovered in the washings (less than 4 per cent of 0.01  $\mu\text{mole per gm}$  of mitochondrial protein, in three separate experiments) was originally at the active center. The effect of  $\text{Cd}^{++}$  is also reversed by  $\text{Mn}^{++}$ ,  $\text{Co}^{++}$ , or  $\text{Ni}^{++}$ . However, in mitochondria inactivated by digestion in phosphate at 30° (14), only  $\text{Mn}^{++}$  and not  $\text{Co}^{++}$  or  $\text{Ni}^{++}$  will restore the activity. This difference in specificity may indicate that, in reversal of inhibition by  $\text{Cd}^{++}$ , the effect is indirect, perhaps, by heterogeneous complex formation which involves both the added metal and  $\text{Cd}^{++}$ , and an anion in the reaction mixture.

The data obtained so far seem inadequate to stress any particular mechanism for the uncoupling activity of  $\text{Cd}^{++}$  as well as the reversal by BAL or  $\text{Mn}^{++}$ . The approach indicated here may be more useful when purified preparations of the phosphorylation system are obtained.

#### SUMMARY

Cadmium ions at as low a concentration as  $5 \times 10^{-6}$  M completely uncouple phosphorylation associated with the oxidation of succinate and of citrate in mitochondria. Ethylenediaminetetraacetic acid, dithiols,  $\text{Mn}^{++}$ ,  $\text{Co}^{++}$ , and  $\text{Ni}^{++}$  reverse the effect of  $\text{Cd}^{++}$ . The bound cadmium is not

removed by repeated washing Attempts to isolate the  $\text{Cd}^{++}$ -binding component with radioactive  $\text{Cd}^{115}$  are described

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# LACTIC DEHYDROGENASE

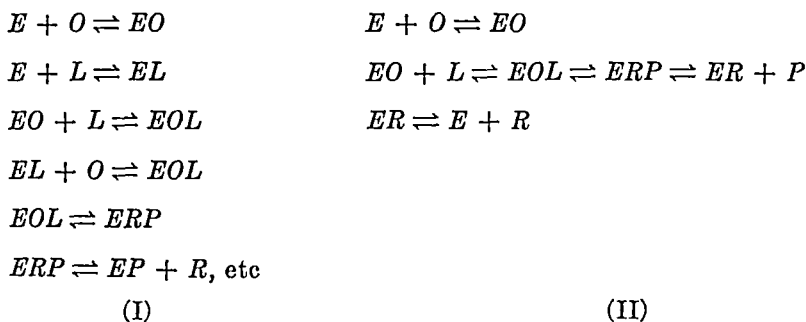
## III MECHANISM OF THE REACTION\*

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In a preceding publication (1) it was concluded that the reaction catalyzed by the lactic dehydrogenase of heart muscle,<sup>1</sup> lactate + DPN  $\rightleftharpoons$  pyruvate + DPNH, can be represented by one of the following reaction sequences



where  $E$  is the enzyme,  $O$  and  $R$  are oxidized and reduced DPN, respectively,  $L$  and  $P$  are lactate and pyruvate, respectively, and  $EO$ ,  $EL$ , etc., are enzyme-substrate complexes. This conclusion was based, in part, upon the fit of the kinetic and equilibrium constants to the relations derived by Alberty (2) for various postulated reaction mechanisms, and in part upon various arguments for the existence of at least one ternary complex in the reaction sequence.

The derivation of the kinetic expression for sequence I is based upon the assumption that the rates of interconversion of  $EOL$  and  $ERP$  are limiting

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<sup>1</sup> The following abbreviations have been used: PCMB, *p*-chloromercuribenzoate, DPN, diphosphopyridine nucleotide, DPNH, reduced diphosphopyridine nucleotide, LDH, lactic dehydrogenase.

and that the dissociation of a reactant from a ternary complex, *e g* the dissociation of *L* from *EOL*, is characterized by a dissociation constant different from that which describes the dissociation of the same reactant from a binary complex, *i e*, the dissociation of *L* from *EL* (2). The derivation of the kinetic expression for sequence II, with use of the steady state approach, assumes a compulsory order of binding of the reactants to the enzyme. In a sense, sequence II is the limiting case of sequence I in which the dissociation constants of the *EL* and *EP* complexes are infinitely large. Although the kinetic results obtained in this laboratory (1) could also be interpreted in terms of the compulsory pathway mechanism in which lactate or pyruvate must be bound by LDH<sup>1</sup> before DPN or DPNH could be bound, this mechanism would not be consistent with the spectroscopic evidence, presented by Chance and Neilands (3), which indicates that an LDH-DPNH complex does exist.

The most direct approach to the problem of whether the reaction is represented by sequence I or sequence II would be to determine, by measurements of equilibrium-binding, which of the remaining possible binary complexes, *EO*, *EL*, or *EP*, is actually formed. Clearly, if *EO* is formed from LDH and DPN, while *EL* and *EP* are not formed from LDH and the corresponding substrates, the reaction must be represented by sequence I. Alternatively, if *EO*, *EL*, and *EP* can all be detected, the possibility of non-specific ion binding must be eliminated before a clear conclusion can be drawn. This paper presents the results of measurements of such binding. Since sulfhydryl groups have been postulated to be responsible for the binding of pyridine nucleotides by dehydrogenases, the binding measurements have been compared with the effects of DPNH and of pyruvate in protecting the enzyme against inactivation by PCMB.

## EXPERIMENTAL

### *Materials*

*Enzyme*—Crystalline lactic dehydrogenase was prepared by a procedure which involves alcohol fractionation and partial acid denaturation in place of the adsorption on calcium phosphate and partial acetone denaturation described by Straub (4). In our hands, this procedure gives better yields of crystalline enzyme than does that of Straub.<sup>2</sup> The conditions for crystallization are those described by Straub (4), the crystalline form and specific enzymatic activity being identical with those obtained in his procedure. Further, crystalline LDH, prepared by this modified procedure, exhibits two electrophoretic components of the same mobility, and in the same relative amounts, as are shown by LDH prepared by the method of Straub (5, 6).

<sup>2</sup> Details of this procedure will be published.

Preliminary estimations of substrate and coenzyme binding and of sulfhydryl reactivity were made with crystalline LDH. Final determinations of DPN binding and of sulfhydryl reactivity were made with the electrophoretically isolated component of higher mobility (1), designated, according to usage of Neilands (6), as Fraction A. In the cases in which no binding was found, measurements were not repeated with Fraction A.

#### *Reagents—*

The preparations of DPN, DPNH, potassium pyruvate, and sodium lactate used in these measurements have been described (1).

PCMB was a product of the Sigma Chemical Company, and its purity, as determined by the procedure of Boyer (7), was 95.2 per cent. If concentrations were corrected by this factor, the molar absorptivity index at  $232\text{ m}\mu$  was identical with that reported by Boyer (7).

#### *Methods*

Studies of equilibrium binding with pyruvate, lactate, and DPN were carried out by the ultracentrifugal separation technique of Velick, Hayes, and Harting (8). 3 ml. volumes of solutions containing LDH and varying concentrations of DPN, pyruvate, or lactate in 0.1 M phosphate buffer, pH 6.80, were centrifuged for 1.5 to 3.5 hours in a mean relative centrifugal field of approximately  $180,000 \times g$ . At the end of this period, 1 to 1.5 ml. of the essentially protein-free supernatant layer was removed from each tube for analysis for DPN, pyruvate, or lactate.

The spectra of LDH and of DPN were shown to be strictly additive. Therefore, the concentration of DPN in the supernatant solutions was determined by measuring their optical density, or that of appropriate dilutions of these solutions, at  $5\text{ m}\mu$  intervals between 250 and  $270\text{ m}\mu$  with the Beckman model DU spectrophotometer. Five simultaneous equations in two unknown concentrations were set up by equating the observed optical density at each wave length to the sum of the products of the known absorptivity indices for DPN and LDH at each wave length and the unknown concentrations of DPN and LDH. These equations were solved in pairs for DPN concentration by using the equation for  $260\text{ m}\mu$ , the wave length of maximal absorption of DPN, as one member of each pair. The mean of the four results was used for the estimation of the binding constants. The average deviation of a single determination from the mean was 3 per cent. The absorptivity indices of DPN and of LDH were taken from tracings made with a Beckman model No. DK-2 recording spectrophotometer with  $18.0 \times 10^3\text{ cm. per mole per liter}$  as the absorptivity index of DPN at  $260\text{ m}\mu$  (9), and  $19.3 \times 10^4\text{ cm. per mole per liter}$  as the absorptivity index of LDH at  $280\text{ m}\mu$ . The latter value was derived from the specific absorptivity reported previously (1) and from Neilands' value of 135,000 for the molecular weight of the enzyme (10).



Preliminary experiments indicated that, in solutions containing high concentrations of both LDH and DPN, each component is sufficiently stable, under the conditions of pH and temperature used for the determinations, that no correction for instability is necessary. The decrease in concentration of DPN in the supernatant layer due to sedimentation during centrifugal separation was found to be about 3 per cent of the initial DPN concentration. Since this is the order of uncertainty of the DPN determination, no correction was made for the change in DPN concentration resulting from sedimentation.

Determinations of pyruvate binding were made only with crystalline LDH and were not repeated with Fraction A. Traces of protein in the supernatant layer were precipitated by the addition of 2 ml of 20 per cent trichloroacetic acid to 15 ml of supernatant solution. The resulting minute precipitate was removed by centrifugation for 22 minutes at  $11,000 \times g$  at  $0^\circ$ . The concentration of pyruvate in this solution was determined by the "total hydrazone" procedure of Friedemann and Haugen (11). Absorbancies were measured in the Bausch and Lomb Spectronic 20 spectrophotometer at  $440 \text{ m}\mu$ .

The determination of lactate binding was also made only with crystalline lactic dehydrogenase. 1 ml of 10 per cent trichloroacetic acid was added to 1 ml of supernatant solution from the ultracentrifugal separation. The resulting solutions were allowed to stand at room temperature for 30 minutes before the trace precipitate was removed by centrifugation for 15 minutes at  $3800 \times g$ .<sup>3</sup> Lactate concentrations in these solutions were determined by the method of Barker and Summerson (12). Absorbancies were measured in the Spectronic 20 spectrophotometer at  $570 \text{ m}\mu$ .

Studies of the reaction of PCMB with LDH were made by the spectrophotometric procedure of Boyer (7). Determinations of enzyme activity, carried out to establish the existence of a protective effect of the coenzymes and substrates, were made at  $25^\circ$  by measuring the rate of disappearance of the  $340 \text{ m}\mu$  absorption, characteristic of DPNH, in the presence of pyruvate.

### *Results*

The results of two series of determinations of the binding of DPN by electrophoretically isolated Fraction A are shown in Fig 1, in which  $r$ , the number of moles of DPN bound per mole of LDH, is plotted against the ratio of  $r$  to the concentration of free DPN in the supernatant solution.

<sup>3</sup> Since the Barker and Summerson procedure for the estimation of lactate (12) involves a further deproteinization step, the methods employed for the deproteinization of supernatant solutions used for the estimation of lactate were somewhat less stringent than those employed with solutions in which pyruvate was estimated.

In a plot of this type (13), the intercept on the axis of ordinates is the maximal number of binding sites, and the negative slope of the line defines the dissociation constant of the LDH DPN complex. In the present case, treatment of the analytical results according to the method of least squares yields a value of  $3.6 \pm 0.4$  for the maximal number of binding sites and  $3.9 \pm 0.7 \times 10^{-4}$  M for the intrinsic dissociation constant of the complex. The absence of curvature in the plot indicates that, within the rather large error of estimation, the affinities of the binding sites for DPN are identical.

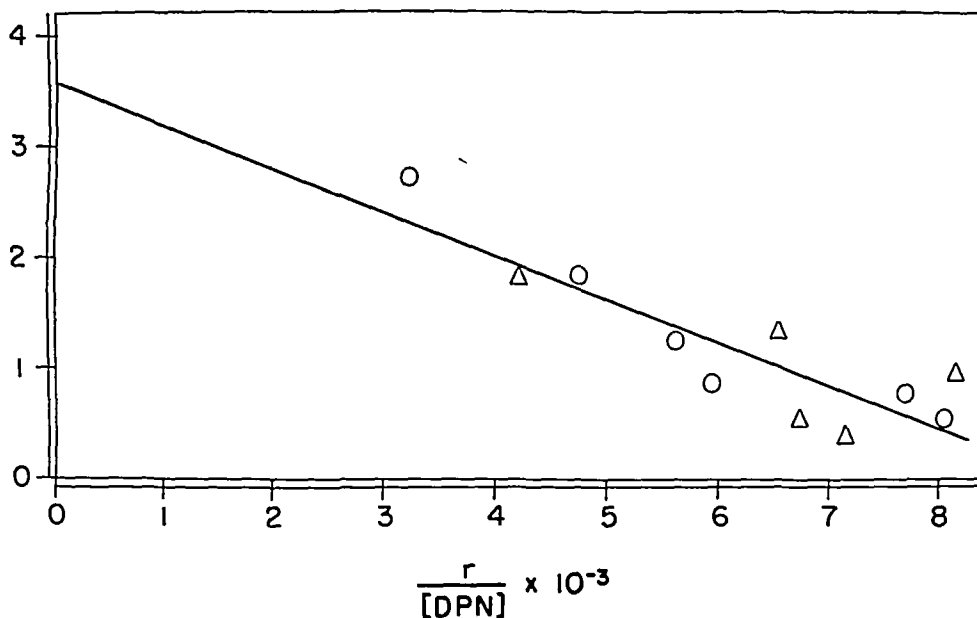


FIG. 1. Plot used for the estimation of the number of binding sites and the magnitude of the dissociation constant of the LDH DPN complex. O, initial LDH concentration of  $6.75 \times 10^{-5}$  M,  $\Delta$ , initial LDH concentration of  $3.64 \times 10^{-5}$  M. The average temperature during each centrifugal separation was within a few tenths of a degree of  $25^\circ$ .

This treatment of the data assumes that the enzyme preparation is free from residual coenzyme. The observations that a solution of the crystalline enzyme, containing 1.25 mg of enzyme per ml, has almost no absorption at  $340 \text{ m}\mu$  and shows no evidence of any discrete absorption peak in this spectral region, and that the addition of sodium-L-lactate to a final concentration of 0.01 M causes no change in the spectrum of the enzyme at either  $340$  or  $260 \text{ m}\mu$ , indicate that this assumption is valid.

Recently, Kaplan, Ciotti, Stolzenbach, and Bachur (14) have reported that highly purified commercial preparations of DPN contain 10 to 15 per cent of the  $\alpha$  isomer of DPN. Since it has been found that, when the DPN used for these measurements is reduced with hydrosulfite, about 10.5

per cent of the absorption at 340  $m\mu$  does not disappear in the presence of LDH and excess pyruvate (1), it seems possible that this figure represents the concentration of  $\alpha$  isomer in the sample of DPN used. If it is assumed that the  $\alpha$  isomer is not bound by the enzyme, the maximal number of binding sites becomes  $3.5 \pm 0.4$  and the value of the dissociation constant  $3.2 \pm 0.6 \times 10^{-4}$  M.

Several attempts were made to demonstrate the binding of pyruvate by crystalline LDH, the results of one such attempt being shown in Table I. The last two columns of Table I indicate the pyruvate concentrations which would be found in the supernatant solution if there were one binding site per mole of LDH, and if the dissociation constant were equal to the

TABLE I  
*Attempt to Demonstrate Binding of Pyruvate by Lactic Dehydrogenase\**

Initial pyruvate concentration	Final pyruvate concentration	Final pyruvate concentration which would have been found for dissociation constant having value	
		$1.5 \times 10^{-5}$ M	$2.5 \times 10^{-3}$ M
$M \times 10^4$	$M \times 10^4$	$M \times 10^4$	$M \times 10^4$
9.75	9.95	8.42	9.40
7.80	7.55	6.48	7.52
4.88	4.78	3.57	4.66
2.93	2.95	1.68	2.78
1.95	2.03	0.793	1.85
0.975	1.00	0.202	0.925

\* The initial LDH concentration was  $1.36 \times 10^{-4}$  M. The mean temperature of the centrifugal separation was 28.80°. The ultracentrifugal separation was carried out for 3.5 hours.

Michaelis constant at 25°,  $1.5 \times 10^{-5}$  M, or if the dissociation constant were equal to the constant characterizing the inhibition of LDH by high concentrations of pyruvate,  $2.5 \times 10^{-3}$  M (1). Since the supernatant layer from control tubes containing enzyme and buffer gave the same optical density reading in the pyruvate determination as did the supernatant layer from tubes containing only buffer, the enzyme preparation appeared to be free from pyruvate.

The results of a single attempt to demonstrate the binding of L-lactate by crystalline LDH are given in Table II. The standard curve for the estimation of lactate concentration was determined from measurements made on the supernatant solutions from tubes which contained no enzyme. Since supernatant solutions from a tube containing enzyme and buffer and from a tube containing only buffer gave identical optical density readings

in the lactate determination, the enzyme preparation was presumed to be free from lactate

Tables I and II indicate that neither pyruvate nor lactate is measurably bound by LDH. Both sets of data indicate a somewhat higher concentration of substrate in the supernatant layers from tubes containing enzyme than that found in the supernatant layers from tubes containing only substrate. Since the enzyme preparation appears to be free from both substrates, it is possible that a portion of this effect may arise from the sedimentation of the enzyme protein which, with its hydration shell, is not a solvent for substrate, a relatively more concentrated substrate solution being left behind in the supernatant layer. A second cause may be that the sedimentation of substrate is hindered by the density gradient arising at the protein concentration gradient, with the result that the av-

TABLE II

*Attempt to Demonstrate Binding of L-Lactate by Lactic Dehydrogenase\**

Initial L-lactate concentration	Final lactate concentration
$M \times 10^4$	$M \times 10^4$
30	30.9
10	10.2
5	5.2
2	2.3

\* The initial lactic dehydrogenase concentration was  $6.86 \times 10^{-6} M$ . The mean temperature during the ultracentrifugal separation was  $24.37^\circ$ . The ultracentrifugal separation was carried out for 2 hours 10 minutes.

erage concentration of substrate in the supernatant layer at the end of the sedimentation process is higher than that in the corresponding layer from tubes containing no protein.

If credence is given to the hypothesis that sulfhydryl groups are involved in the transfer of electrons from lactate to DPN (15) or in the binding of coenzyme to enzyme (16), the present observation that 3 to 4 molecules of DPN are bound to 1 mole of enzyme appears to be inconsistent with Neilands' report that approximately 2 moles of sulfhydryl groups per mole of enzyme are capable of reaction with PCMB (10). An attempt was made to clarify this point.

Boyer (7) has reported that the change in absorbancy at  $250 m\mu$ , resulting from mercaptide formation with PCMB, varies somewhat with the source of the sulfhydryl group. Therefore, measurements were made of the increase in absorbancy in the presence of limiting quantities of mercuribenzoate. The increase in absorbancy accompanying mercaptide formation with LDH was found to be  $7.2 \times 10^3 \text{ cm}^2$  per mole per liter.

Several determinations of the number of reactive sulfhydryl groups were made in the presence of excess mercuribenzoate. A typical result is shown

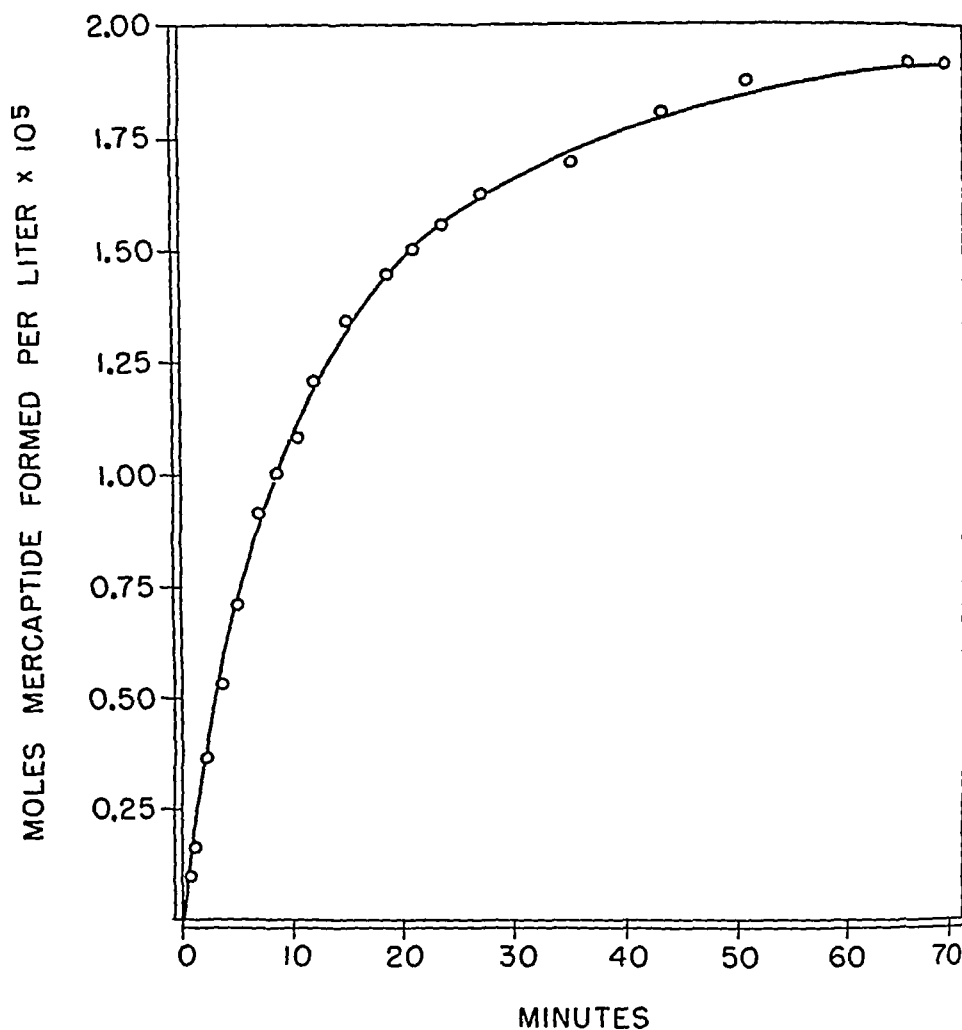


FIG. 2. Plot of the course of the reaction of  $2.87 \times 10^{-5}$  M PCMB with  $4.48 \times 10^{-5}$  M LDH at  $25^\circ$  in 0.1 M phosphate, pH 6.80. Observed absorbancy changes with time were converted to changes in mercaptide concentration by using  $7.2 \times 10^3$  cm per mole per liter as the value of the absorbancy index change. The curve is a calculated second order rate curve for an assumed initial concentration of rapidly reacting sulfhydryl groups of  $1.97 \times 10^{-5}$  M and for a rate constant of 61.4 liters per mole per second.

in Fig. 2. The estimation of the number of reactive groups is complicated by the fact that, after the reaction (Fig. 2) appears to be essentially complete, a slow increase in absorbancy continues, approximately linearly with time, for a long period. An approximate value for the number of rapidly reacting sulfhydryl groups was obtained by making use of Boyer's

observation that the reaction of egg albumin and of  $\beta$ -lactoglobulin with PCMB follows second order kinetics (7). The value used for the initial concentration of rapidly reacting sulfhydryl groups was adjusted by trial until a plot of the logarithm of the ratio of concentrations of the two reactants against time gave a good fit to the portion of the curve shown in Fig. 2. From these data it was calculated that the initial concentration of rapidly reacting groups was  $1.97 \times 10^{-5}$  M, and that the rate constant was 61.4 liters per mole per second. The curve was calculated from these constants. Since the lactic dehydrogenase concentration was  $4.48 \times 10^{-6}$  M, it appears that there are 4.4 moles of sulfhydryl groups per mole of enzyme which react rapidly and homogeneously with mercuribenzoate. Since the slow reaction continues at a rate which indicates the formation of approximately 0.25 mole of mercaptide per mole of enzyme per hour, it seems probable that the number of rapidly reacting groups is four per mole of LDH. The question whether the sulfhydryl groups which react with PCMB are protected by DPNH was approached by incubating two series of solutions,  $1.46 \times 10^{-7}$  M, with respect to LDH, for approximately 5 hours at room temperature, with concentrations of PCMB varying between 0.68 and  $17.1 \times 10^{-5}$  M. One series of solutions was  $9.62 \times 10^{-5}$  M with respect to DPNH, while the other series contained no DPNH. The solvent was 0.1 M phosphate, pH 6.80. At the end of the incubation period, an appropriate dilution of each solution was tested for enzymatic activity by measuring the reaction rate from the pyruvate side of the reversible reaction. A small protective effect of DPNH was found, even at the highest concentration of PCMB. When the concentration of PCMB was of the order of  $10^{-5}$  M, 50 per cent of the activity was lost from solutions containing no DPNH and only about 10 per cent of the activity was destroyed in solutions containing DPNH.

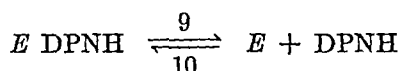
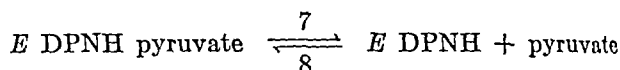
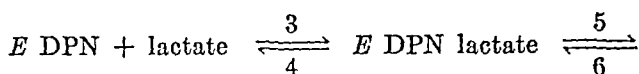
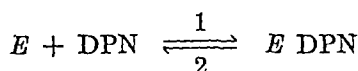
In a similar experiment, in which  $5.77 \times 10^{-7}$  M LDH was incubated with  $4.0 \times 10^{-6}$  M PCMB in the presence and absence of  $1.25 \times 10^{-3}$  M pyruvate for 20 minutes, no protection of the enzyme by pyruvate could be demonstrated.

Although sulfhydryl groups appear to be necessary to the activity of the enzyme, it had previously been shown that long standing in dilute solution in air at neutral pH values does not inactivate the enzyme (1). It was further observed that dialysis of the enzyme against 0.01 M cysteine at pH 7.0 for 24 hours in the cold does not enhance the activity of the enzyme.

#### DISCUSSION

The findings that DPN is bound to the enzyme while pyruvate and lactate are not, and that DPNH protects the enzyme against inactivation

by PCMB while pyruvate does not, and the spectroscopic demonstration by Chance and Neilands (3) of the existence of an LDH DPNH complex are all consistent with the representation of the reaction as



The strongest evidence in favor of the mechanism shown is the failure to observe the binding of pyruvate by LDH. While it can be argued that this failure to observe binding is a measure of the sensitivity of the pyruvate determinations rather than an indication that pyruvate is not bound, the present measurements appear to rule out reaction sequence I as a possible mechanism. This conclusion is based upon the fact that, if sequence I were the mechanism, the Michaelis constants for pyruvate and DPNH,  $K_P$  and  $K_R$ , respectively, would be related to the dissociation constants of the LDH pyruvate and LDH DPNH complexes,  $K_P'$  and  $K_R'$ , and to the complex constant  $K_{PR}$  by the relation (2, 17),  $K_P K_R' = K_P' K_R = K_{PR}$ , so that  $K_{PR}/K_R = K_P'$ . If the values found for  $K_P$ ,  $K_R$ , and  $K_{PR}$  at 25° are substituted into this expression,  $K_P'$  is found to be  $4 \times 10^{-6}$  M. Since sequence I thus requires the dissociation constant of the LDH pyruvate complex to be less than the Michaelis constant for pyruvate, and since, as shown in Table I, binding characterized by such a small dissociation constant could be detected with a measure of precision, it is considered highly improbable that sequence I represents the reaction.

If the same considerations are applied to the reverse reaction, it follows that the ratio  $K_{OL}/K_O$  yields a value of approximately  $6 \times 10^{-3}$  M for  $K_L'$ , the dissociation constant of the LDH lactate complex. It is apparent that binding, characterized by a dissociation constant of this magnitude, would be impossible to detect with the methods available for the estimation of lactate, and that relatively little reliance can be placed on the failure to detect lactate binding.

Although the kinetic constants characterizing the lactic dehydrogenase system at a variety of temperatures have been reported previously (1), no meaning could be assigned to the constants because the reaction mechanism was unknown. For the mechanism given above, the kinetic con

stants are defined by<sup>4</sup>

$$\begin{aligned} V_f &= k_5 k_7 k_9 E/F & V_r &= k_2 k_4 k_6 E/H \\ K_O &= k_5 k_7 k_9 / k_1 F & K_R &= k_2 k_4 k_6 / k_{10} H \\ K_L &= k_9 G / k_3 F & K_P &= k_2 G / k_8 H \\ K_{OL} &= k_2 k_9 G / k_1 k_3 F & K_{PR} &= k_2 k_9 G / k_8 k_{10} H \end{aligned}$$

where  $V_f$  and  $V_r$  are the maximal reaction velocities in the forward and reverse directions, respectively,  $K_O$ ,  $K_R$ ,  $K_L$ , and  $K_P$  are the Michaelis constants for oxidized and reduced DPN, lactate, and pyruvate, respectively, and  $K_{OL}$  and  $K_{PR}$  are complex constants which have the property that  $K_{OL}/K_L = k_2/k_1$ , the dissociation constant of the LDH DPNH complex.  $E$  is the total enzyme concentration,  $F$  is defined<sup>4</sup> by  $(k_9(k_6 + k_7) + k_5(k_7 + k_9))$ ,  $G = k_4 k_6 + k_4 k_7 + k_5 k_7$ , and  $H = k_2(k_4 + k_5) + k_6(k_2 + k_4)$ . Although a marked simplification can be made in these rather formidable definitions if it is assumed that one of the ternary complexes is much more stable than the other (2), there appears to be no present basis for this assumption.

The value found<sup>5, 6</sup> for the ratio  $K_{OL}/K_L$  at 25° is  $2.9 \pm 0.4 \times 10^{-4}$  M. While the agreement of this calculated value of  $k_2/k_1$  with that found by direct measurement,  $3.2 \pm 0.6 \times 10^{-4}$  M, may be fortuitous, in view of the considerable experimental error which attends the estimation of each of these parameters, and in view of the unknown effect of the hydrostatic pressure developed during the ultracentrifugal separation upon the equilibrium being measured, this measure of agreement does appear to justify the extension of the calculation of  $k_2/k_1$  to other temperatures at which the kinetic constants have been estimated. Table III indicates the variation of the calculated dissociation constants of the LDH DPN and LDH DPNH complexes with temperature over the range from 15–35°.

<sup>4</sup> There is an error in the previous definition of these constants (2). The present definitions, which have been checked by Dr R. A. Alberty, enable the conclusion to be drawn that, for those mechanisms in which constants of the type  $K_{AB}$  and  $K_{CD}$  appear,  $K_{AB}/K_B$  is equal to the dissociation constant of the EA complex and  $K_{CD}/K_D$  is equal to the dissociation constant of the EC complex.

<sup>5</sup> Since the kinetic constants for the two sides of the reversible reaction were not determined at identical temperatures, and since there is no independent way to estimate the errors of these determinations, the values used here were determined from the least square lines relating the logarithms of the various constants to reciprocal absolute temperature. The standard errors of these calculated values were taken to indicate the internal precision of the determinations.

<sup>6</sup> No correction was made in the values of  $K_O$  and  $K_{OL}$  for the concentration of the  $\alpha$  isomer of DPN. The effect of the non-reactivity of the  $\alpha$  isomer would be to reduce the values given by about 10 per cent. The effect on the binding constant is much larger, since the  $\alpha$  isomer is relatively more concentrated in the supernatant solution as a result of the binding of the reactive  $\beta$  isomer by the enzyme.



It is of interest that the calculated dissociation constants for both enzyme-pyridine nucleotide complexes decrease with increasing temperature. Since, for the *dissociation* of either form of the coenzyme from the enzyme, the free energy change is clearly positive and since this dissociation appears to be characterized by a negative enthalpy change, it is clear that the dissociation of the LDH DPNH complex is accompanied by a decrease in entropy. A similar result was found by Velick (18) from the spectrophotometric titration of phosphoglyceraldehyde dehydrogenase with DPN. Klotz (19) has accounted for decreases in entropy which accompany the dissociation of bound ions from proteins as being due to the fact that the increase in entropy accompanying the dissociation process itself is more than offset by the decrease in entropy resulting from electrostriction of solvent by the separate ions.

TABLE III

*Variation of Values of Ratios  $K_{OL}/K_L$  and  $K_{PR}/K_P$  with Temperature\**

Temperature	log $K_{OL}$	log $K_L$	$K_{OL}/K_L$	log $K_{PR}$	log $K_P$	$K_{PR}/K_P$
°C			$\times 10^4 \text{ M per l}$			$\times 10^4 \text{ M per l}$
15	-6.81 ±0.08	-3.66 ±0.04	7.0 ±1.6	-11.37 ±0.08	-5.36 ±0.03	9.8 ±2.2
25	-6.73 ±0.05	-3.19 ±0.03	2.9 ±0.4	-10.97 ±0.07	-4.88 ±0.02	8.1 ±1.4
35	-6.65 ±0.07	-2.75 ±0.04	1.3 ±0.3	-10.60 ±0.11	-4.43 ±0.03	6.8 ±2.0

\* See foot-note 6 of the text

It is apparent from Table III that extrapolation of the values found for  $K_{PR}/K_P$  to 5° yields a value for  $k_9/k_{10}$  of about  $10^{-6} \text{ M}$ . Chance and Neilands (3), using crystalline LDH rather than Fraction A, have estimated this dissociation constant to be  $7 \times 10^{-6} \text{ M}$  at 5° on the assumption that there is one binding site per mole of enzyme. In view of the uncertainties accompanying both estimations of the magnitude of this dissociation constant, the agreement between these values appears to be satisfactory.

From the definitions given for the kinetic constants it follows that, if  $V_f$  is expressed as the turnover number per mole of enzyme per second, the ratio  $V_f/K_o$  is equal to  $k_1$ , and the product  $(V_f/K_o)(K_{OL}/K_L)$  equals  $k_2$ . Similar relations apply for the reverse reaction. The values calculated for  $k_1$ ,  $k_2$ ,  $k_9$ , and  $k_{10}$ , respectively are  $6.7 \pm 0.1 \times 10^6$ ,  $1.9 \pm 0.3 \times 10^3$ ,  $1.9 \pm 4 \times 10^2$ , and  $2.3 \pm 0.2 \times 10^8$  reciprocal seconds at 25°. If the four DPN-binding sites are all enzymatically active, the magnitude of each of these constants would be reduced to one-fourth of the value given.

The discrepancy between the present results and those of Neilands (10) for the maximal number of rapidly reacting sulfhydryl groups per mole of LDH may lie in the fact that Neilands used whole crystalline enzyme for his measurements, while the present measurements were made with Fraction A. Preliminary measurements, made with crystalline enzyme, yielded lower values than were found with Fraction A.<sup>7</sup> The finding of three to four binding sites for DPN and the observation that approximately four sulfhydryl groups react rapidly with PCMB suggest that sulfhydryl groups have a role in binding the coenzyme to the enzyme. This suggestion is strengthened by the fact that DPNH protects the enzyme against PCMB inactivation.

According to the proposed reaction scheme, the binding of coenzyme by the enzyme surface generates a binding site for substrate, which did not previously exist. If it is assumed that substrate is bound so that it is superimposed upon at least a portion of the nicotinamide ring of the coenzyme, the direct stereospecific transfer of hydrogen between substrate and coenzyme, demonstrated for LDH by Loewus, Ofner, Fisher, Westheimer, and Vennesland (20), is obviously facilitated. If it is assumed that pyruvate is bound to the enzyme-coenzyme complex at two points, one of which arises in the protein surface and one from the nucleotide portion of the complex, the inhibition by high pyruvate concentrations can be accounted for if it is assumed that a relatively weak LDH pyruvate complex prevents DPNH from forming the LDH DPNH complex which is essential to reaction. The last column of Table I indicates that the direct detection of the LDH pyruvate complex, which is postulated from kinetic data, lies at the limit of precision of the pyruvate determination.

Since the present results indicate the role of the pyridine nucleotides to be different from that of pyruvate and lactate, it appears that the term *coenzyme* connotes the kinetic, as well as the metabolic, function of the pyridine nucleotides more adequately than does the more recent usage of *cosubstrate* in this connection.

#### SUMMARY

Diphosphopyridine nucleotide (DPN) has been found to be bound at approximately four sites on the lactic dehydrogenase molecule. The dissociation constant of the enzyme DPN complex is of the order of  $3 \times 10^{-4}$  M.

Pyruvate and lactate are not bound by lactic dehydrogenase to a measurable extent.

<sup>7</sup> After this manuscript was submitted for publication, Nygaard (21) published the finding that crystalline LDH, containing both fractions A and C, contains 2.9 PCMB-reactive sulfhydryl groups per mole. Measurements made in this laboratory agree well with this value.

Approximately 4 moles of sulfhydryl groups per mole of enzyme react rapidly with mercuribenzoate. The inactivation of the enzyme by mercuribenzoate is inhibited by reduced diphosphopyridine nucleotide but not by pyruvate.

These results indicate that the enzyme forms a complex with one form of the coenzyme and that the resulting binary complex then forms a ternary complex with the substrate. Previously reported kinetic data (1) have been interpreted in terms of this mechanism, and the dissociation constants of the enzyme-coenzyme complexes have been calculated from the kinetic results. The order of magnitude of the individual rate constants for the reaction between enzyme and the coenzymes has been indicated.

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# THE ENZYMATIC CONVERSION OF ANTHRANILIC ACID TO INDOLE\*

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Studies with various microorganisms have implicated anthranilic acid and indole as intermediates in the biosynthesis of tryptophan (1-5). The mechanism of tryptophan synthesis from indole has been established (6-9), but relatively little is known about the conversion of anthranilic acid to indole. Isotope studies on the synthesis of indole from anthranilic acid have shown that the carboxyl carbon of anthranilic acid is lost during this conversion (10, 11) and that the 2 carbon atoms which complete the pyrrole ring of indole are probably derived from C-1 and C-2 of a ribose derivative (11). Enzymatic investigations have implicated 5-phosphoribosyl-1-pyrophosphate<sup>1</sup> as the actual source of the 2 carbon atoms and have led to the isolation of an intermediate, indole-3-glycerol phosphate, in the conversion of anthranilic acid to indole (12). The present report is concerned with the enzymatic conversions of anthranilic acid to indole-3-glycerol phosphate and of indole-3-glycerol phosphate to indole.

## Methods

A tryptophan auxotroph of the K-12 strain of *Escherichia coli*, strain T-3, was employed as the source of the enzymes catalyzing the conversion of anthranilic acid to indole. This mutant is blocked in the synthesis of anthranilic acid, and thus its tryptophan requirement can be satisfied by anthranilic acid or indole. The conditions of growth used and the method of preparation of crude extracts of this mutant were essentially the same as described previously (11). The mutant was grown on a minimal medium (13), supplemented with anthranilic acid (2  $\gamma$  per ml) and 0.16 per cent glucose. Cultures were incubated with shaking for 40 hours at 30°. The cells were harvested by centrifugation, washed once with saline, and suspended in 0.1 M phosphate buffer at pH 7.0. These washed suspensions were then disrupted in a 9 kc Raytheon sonic oscillator and centrifuged.

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<sup>1</sup> The following abbreviations are employed in this paper: IGP, indole 3-glycerol phosphate, IG, indole-3-glycerol, PRPP, 5-phosphoribosyl-1-pyrophosphate, ATP, adenosine triphosphate, ADP, adenosine diphosphate, R5P, ribose-5-phosphate, P<sub>i</sub>, inorganic phosphate, Tris, tris(hydroxymethyl)aminomethane.

twice to remove debris for 20 minute periods at  $60,000 \times g$  in a Spinco preparative centrifuge. The supernatant solutions obtained contained approximately 35 mg of protein per ml. These crude extracts, when suitably supplemented, catalyzed the complete conversion of anthranilic acid to indole (14). Ammonium sulfate fractionation of such preparations yielded two separate fractions, arbitrarily designated Fractions A and B, which catalyzed successive reactions in the transformation of anthranilic acid to indole.

Fraction A was prepared as follows: 18.7 gm of solid ammonium sulfate were added for each 100 ml of crude centrifuged extract of strain T-3. The mixture was allowed to stand in an ice bath for 20 minutes and then was centrifuged in the cold. The supernatant solution was saved for the isolation of Fraction B, while the precipitate was used in the further preparation of Fraction A. The precipitate was dissolved in 0.1 M phosphate at pH 7.0 (30 ml for every 100 ml of extract) and dialyzed, with internal stirring, for 3 hours against 0.02 M phosphate at pH 7.8. The dialyzed preparation was treated with ammonium sulfate (4.3 gm for each 40 ml) and, after 20 minutes, was centrifuged in the cold. The precipitate was discarded and 3 gm of ammonium sulfate were added to the supernatant solution. After 20 minutes, the precipitate was collected by centrifugation and dissolved in 15 ml of 0.1 M phosphate buffer at pH 7.8. It was then dialyzed for 3 hours against 0.02 M phosphate buffer at pH 7.8 and stored at  $-15^{\circ}$ . This preparation, designated Fraction A, catalyzes the conversion of anthranilic acid to IGP and is unable to convert the latter compound to indole.

The supernatant solution from the first ammonium sulfate precipitation was treated with an additional 9.5 gm of ammonium sulfate and the precipitate collected by centrifugation. This precipitate was dissolved in 30 ml of 0.1 M Tris buffer at pH 7.8 and dialyzed against the same buffer (0.02 M) for 3 hours. The preparation obtained was then placed in a  $50^{\circ}$  water bath and stirred continuously for 20 minutes. After this treatment, the mixture was chilled rapidly and the precipitated protein removed by centrifugation. The supernatant solution was treated with ammonium sulfate (8.6 gm for every 30 ml) and, after standing for 20 minutes, was centrifuged. The precipitate obtained was dissolved in 0.1 M Tris buffer at pH 7.8 and dialyzed for 3 hours against the same buffer (0.02 M). The final preparation, designated Fraction B, was stored at  $-15^{\circ}$ . This fraction converts IGP to indole but does not catalyze the utilization of anthranilic acid.

#### *Assay Methods*

Disappearance of anthranilic acid was followed fluorometrically (15). Aliquots (0.05 or 0.1 ml) were added to 1.0 ml of 0.1 M phosphate buffer

at pH 6.0 and read in a Farrand fluorimeter. The assay range employed was 0.2 to 5  $\gamma$  of anthranilic acid.

Indole was determined colorimetrically with Ehrlich's reagent (16) and pentose by the procedure of Umbreit *et al* (17). Inorganic phosphate was determined by the method of Fiske and Subbarow (18).

IGP was assayed in several ways: qualitatively with a modified ferric chloride Salkowski reagent (19) or by enzymatic conversion to indole, or quantitatively by extinction measurements at 280  $m\mu$  or by oxidation with metaperiodate to indole-3-aldehyde. These methods are described below.

The ferric chloride assay was performed as follows. To 1 ml containing IGP or IG, 1.5 ml of ferric chloride reagent (1 ml of 0.5 M  $\text{FeCl}_3$  plus 50 ml of water plus 30 ml of concentrated  $\text{H}_2\text{SO}_4$ ) were added. A pink to red color developed in about 3 minutes. As little as 10  $\gamma$  of IGP could be detected by this method. The intensity of the color produced was proportional to IGP and IG concentration, but the color did not persist in the presence of high salt concentrations. Nevertheless, this method had the advantage of being rapid and thus was employed in the isolation of IGP.

IGP was assayed enzymatically as follows. 0.1 to 0.3 ml (5 to 50  $\gamma$ ) of a solution of IGP was added to 0.2 ml of 0.1 M phosphate at pH 6.0. An excess of Fraction B (0.05 ml) was then added and the mixture incubated at 37° for 15 minutes. The reaction was terminated by addition of 0.1 ml of 1 N NaOH and the indole formed was extracted with 2 ml of toluene. An indole assay was performed by using Ehrlich's reagent on a 1 ml aliquot of the toluene layer. Between 80 and 95 per cent of IGP was converted to indole by this method.

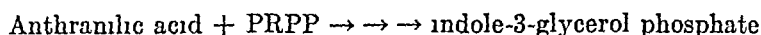
Neutral or alkaline aqueous solutions of IGP, when free from other absorbing materials, were standardized in a Beckman spectrophotometer at 280  $m\mu$ . The 280/260 and 280/240 ratios (1.32 and 3.1, respectively, for IGP) were used to assess the purity of IGP during isolation.

The preferred method of IGP assay was based on oxidation to indole-3-aldehyde as follows. 0.1 ml of 1 M acetate buffer at pH 5.0 and 0.5 ml of 0.1 N sodium metaperiodate were added to 0.4 ml of a solution of IGP and the mixture was incubated at room temperature for 20 minutes. 0.25 ml of 1 N NaOH was then added and the indole-3-aldehyde formed was extracted with 5 ml of ethyl acetate. The ethyl acetate layer was clarified by a 1 minute centrifugation, and its indole aldehyde content was determined in a Beckman spectrophotometer at 290  $m\mu$ . An IGP sample, previously standardized by absorption at 280  $m\mu$ , and an indole-3-aldehyde sample were similarly treated and employed as standards. If the IGP sample contained indole, the indole was first extracted with 3 ml of toluene. Aliquots of the aqueous layer were then removed for IGP assay.

The aldolase employed was purchased from the Nutritional Biochemicals Corporation.

### Results

*Conversion of Anthranilic Acid to Indole Glycerol Phosphate*—Following separation of the enzymes involved in the conversion of anthranilic acid to indole into two fractions, the characteristics and requirements of the fractions were investigated. Fraction A catalyzes the following interconversion



The requirements for this sequence of reactions are shown in Table I. It can be seen in Experiment A, Table I, that PRPP and  $\text{Mg}^{++}$  ions are re-

TABLE I  
Requirements for Anthranilic Acid Utilization

Experiment A			Experiment B		
Supplements	Disappearance of anthranilic acid		Supplements	Disappearance of anthranilic acid	
	10 min	20 min		10 min	20 min
	$\mu\text{mole}$	$\mu\text{mole}$		$\mu\text{mole}$	$\mu\text{mole}$
Complete system	0.12	0.2	Complete system	0.08	0.14
Minus PRPP	0	0	Minus ATP	0	0
" $\text{Mg}^{++}$	0.02	0.03	" R5P	0	0
			" $\text{Mg}^{++}$	0	0.01

In Experiment A, each tube contained 0.22  $\mu\text{mole}$  of anthranilic acid, 0.1 ml of 0.5 M phosphate buffer at pH 7.8, and 0.03 ml of Fraction A in a final volume of 0.6 ml. PRPP (0.24  $\mu\text{mole}$ ) and  $\text{Mg}^{++}$  (1  $\mu\text{mole}$ ) were added, except as indicated.

In Experiment B, each tube contained anthranilic acid, buffer, and enzyme as above, and, in addition, ATP (1.2  $\mu\text{moles}$ ), R5P (0.5  $\mu\text{mole}$ ), and  $\text{Mg}^{++}$  (1  $\mu\text{mole}$ ), except as indicated.

quired for anthranilic acid utilization. Little or no reaction occurs when either of these supplements is omitted. The requirement for PRPP can be satisfied by supplying both ATP and R5P (Experiment B, Table I), however, with the latter supplements, anthranilic acid disappears at a somewhat lower rate, as is evident from the rate data in Fig. 1. Disappearance of anthranilic acid is related to the amount of pentose supplied, whether this pentose is in the form of R5P or PRPP. In two experiments in which all components except R5P were present in excess, 0.26 and 0.22  $\mu\text{moles}$  of anthranilic acid disappeared when 0.25 and 0.21  $\mu\text{moles}$  of R5P were supplied. In a similar experiment in which 0.24  $\mu\text{mole}$  of PRPP was limiting, 0.2  $\mu\text{mole}$  of anthranilic acid disappeared. Furthermore, in the presence of Fraction A, both pentose (as PRPP) and anthranilic acid disappear at approximately the same rate. This can be seen in Fig. 2. It can also be

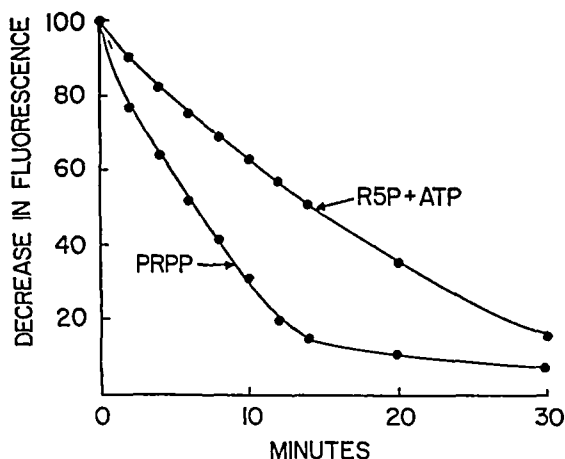


FIG 1 Comparison of the rate of anthranilic acid utilization in the presence of PRPP or ATP plus R5P. Both tubes contained 0.44  $\mu$ mole of anthranilic acid, 100  $\mu$ moles of phosphate at pH 7.8, 2  $\mu$ moles of  $MgSO_4$ , and 0.06 ml of Fraction A. One tube contained 0.96  $\mu$ mole of PRPP while the second contained 1.2  $\mu$ moles of ATP and 0.8  $\mu$ mole of R5P. The final volume was 1.2 ml.

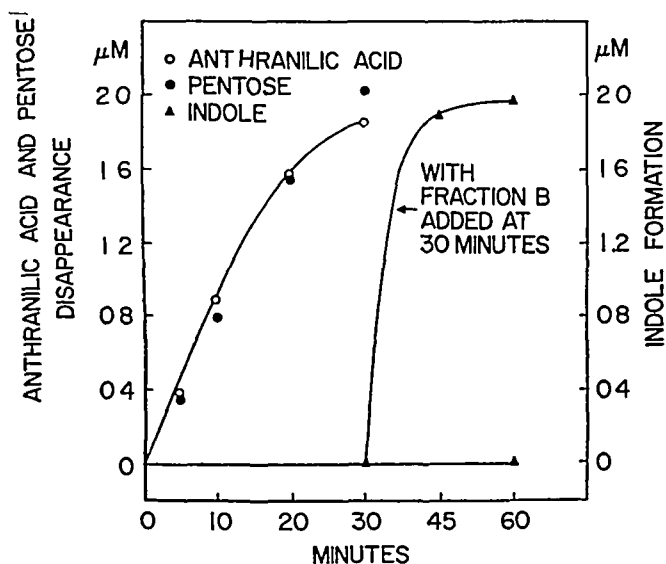


FIG 2 Comparison of anthranilic acid and pentose disappearance and indole formation. The reaction mixture contained 2.2  $\mu$ moles of anthranilic acid, 3.6  $\mu$ moles of PRPP, 1 mmole of phosphate buffer at pH 7.8, 10  $\mu$ moles of  $MgSO_4$ , and 0.3 ml of Fraction A in a final volume of 6 ml. Aliquots were removed at the times indicated and assayed for anthranilic acid, pentose (0.5 ml aliquots were treated with 1 ml of 10 per cent perchloric acid, the precipitate was removed, and portions of the supernatant solution were assayed), or indole.



seen that indole is not formed in the presence of Fraction A but is formed when Fraction B is added to the reaction mixture

In several experiments, disappearance of anthranilic acid was compared with IGP formation, the results of two typical experiments were as follows 0.16 and 0.18  $\mu$ moles of anthranilic acid disappeared, while 0.13 and 0.16  $\mu$ moles of IGP were formed

In addition to the requirements for anthranilic acid utilization, mentioned above, there is an absolute requirement for  $P_i$  when ATP and R5P are substituted for PRPP. With PRPP there is no requirement for  $P_i$ , however, if either ATP or ADP is added to a reaction mixture containing PRPP, anthranilic acid uptake is inhibited. This inhibition is relieved by inorganic phosphate. The experiments described below and performed with  $P^{32}$ -labeled inorganic phosphate have excluded  $P_i$  as a precursor of the phosphate group of IGP. Our present interpretation of these findings is that  $P_i$  relieves the ATP or ADP inhibition of the reaction between anthranilic acid and PRPP.

*Origin of Phosphate Group of Indole Glycerol Phosphate*—Isotope experiments were performed with  $P^{32}$ -labeled  $P_i$  and PRPP-5- $P^{32}$  to determine the origin of the phosphate group of IGP. Fraction A was incubated with labeled PRPP and unlabeled  $P_i$  in the presence of anthranilic acid until most of the anthranilic acid disappeared. The reaction mixture was then heated to precipitate protein, was centrifuged, and the supernatant solution chromatographed on paper, a developing solvent containing methyl alcohol, ethyl acetate, and water (1:2:1 by volume) being used. Identical samples incubated with labeled  $P_i$  and unlabeled PRPP were also chromatographed in this manner. Radioautographs of the chromatograms were prepared and the position and shape of the radioactive spots were compared with IGP spots (developed by spraying the paper with ferric chloride reagent). The results of these experiments clearly showed that the phosphate group of IGP was derived from the terminal phosphate of PRPP and not from  $P_i$ .

*Specificity of Fractions A and B*—A number of substituted anthranilic acids, 3-methylantranilic acid, 4-methylantranilic acid, 5-methylantranilic acid, and 5-fluoroanthranilic acid, were tested as possible substrates for the reaction catalyzed by Fraction A. Incubation mixtures comparable to those in Table I were employed, with the substitution of an equimolar amount of one of the above compounds for anthranilic acid. All of the substituted anthranilic acids except 3-methylantranilic acid were attacked by Fraction A. This is shown in Fig. 3, from which it can also be seen that the reaction proceeded somewhat faster with 5-methyl- or 5-fluoroanthranilic acid as substrate than with anthranilic acid. Following incubation, ferric chloride reagent was added to the reaction mixtures con-

taining the substituted anthranilic acids. All except the one containing 3-methylanthranilic acid gave the color reaction characteristic of IGP. To a similar set of tubes, Fraction B was added and the mixtures were reincubated. The contents of each were then extracted with toluene and aliquots of the toluene layer were assayed for indole-like compounds with Ehrlich's reagent. Positive reactions were given by all except the reaction mixture containing 3-methylanthranilic acid. The indole-like compounds formed were presumably the substituted indoles corresponding to the substituted anthranilic acids employed. It was previously shown that, with cell suspensions of strain T-3, 4-methylanthranilic acid is converted to the corresponding substituted indole, 6-methylindole (20).

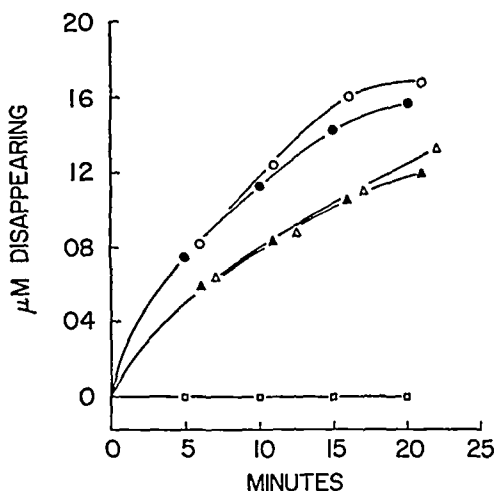


FIG 3 Comparison of the rate of utilization of various substituted anthranilic acids: ○, 5-fluoroanthranilic acid, ●, 5-methylanthranilic acid, ▲, anthranilic acid, △, 4-methylanthranilic acid, □, 3-methylanthranilic acid

*Isolation of Indole-3-Glycerol Phosphate*—The following incubation mixture was employed for the formation of IGP: 0.75 mmole of anthranilic acid, 2 mmoles of ATP, 0.8 mmole of R5P, 1.7 mmoles of  $\text{MgSO}_4$ , 17 mmoles of phosphate buffer at pH 8.2, and 34 ml of Fraction A in a final volume of 1080 ml. The mixture was incubated at  $37^\circ$  for 30 to 40 minutes. Aliquots were removed every 5 to 10 minutes to follow the disappearance of anthranilic acid. At the end of the incubation period the mixture was chilled rapidly and 1 N acetic acid was added until the pH was lowered to 6.5 to 7.0. Several portions of Darco G-60 (acid- and alkali-washed) were then added to adsorb the IGP. After each addition, the Darco was removed by filtration and a portion of the filtrate assayed for IGP with ferric chloride reagent. The Darco treatment was discontinued when a negative test for IGP was obtained. The charcoal was then washed once with water and the IGP eluted by stirring with 40 per cent alcohol containing

5 ml of concentrated  $\text{NH}_4\text{OH}$  per liter The eluate was freed from charcoal by filtration and was then concentrated *in vacuo* to a small volume (20 to 30 ml) This solution was adjusted to pH 8 to 8.5 with saturated  $\text{Ba}(\text{OH})_2$  and a 25 per cent solution of barium acetate was added until further additions no longer produced a precipitate The precipitate was removed by centrifugation and, before being discarded, was washed three times with small volumes (3.5 ml) of water containing a few drops of  $\text{Ba}(\text{OH})_2$  The washings were combined with the original supernatant solution and 1 ml of barium acetate solution was added, followed by 2 volumes of acetone The precipitate was collected by centrifugation and washed twice with acetone The final precipitate contained most of the IGP This precipitate was dissolved in water and applied to a  $2 \times 32$  cm Dowex 1 chloride (2 per cent cross-linked) column The column was prepared by treating Dowex 1 chloride in the column successively with 200 ml of 1 M NaCl (this and all subsequent solutions added to the column contained 0.1 ml of 1 M NaOH per 100 ml) and with 100 ml of 0.01 M NaOH The IGP solution was then applied and the column again treated with 100 ml of dilute alkali It was essential that all solutions applied to the column were alkaline, otherwise the IGP was destroyed during isolation The column was then washed with 600 ml of 0.1 M NaCl The IGP was eluted from the Dowex by gradient elution with 400 ml of 0.1 M NaCl in the mixing flask and 0.5 M NaCl in the reservoir flask 20 ml fractions were collected and a sample from each was tested for IGP with ferric chloride reagent The IGP was usually present in fractions 18 to 30 The purity of these fractions was determined by measuring their absorption at 240, 260, and 280  $\text{m}\mu$  Of the ten to twelve fractions containing IGP, the first three to five usually contained appreciable amounts of adenylic acid, while the last two fractions occasionally contained small amounts of ADP The IGP fractions containing impurities were combined and the Darco and Dowex steps repeated IGP fractions which were free of absorbing impurities were adjusted to pH 6.5 to 7 and passed through a  $2 \text{ cm} \times 5 \text{ cm}$  (diameter) column of Darco G-60 The IGP was adsorbed completely The column was washed with water and finally the IGP was eluted with 40 per cent alcohol containing 1 ml of concentrated  $\text{NH}_4\text{OH}$  per 100 ml The eluate was concentrated *in vacuo* to about 5 ml and 1 N acetic acid was added until the pH was lowered to about 6 This treatment precipitated a small amount of charcoal which always contaminated the eluate The precipitate was removed by centrifugation and the supernatant solution adjusted to pH 8 to 8.5 with saturated  $\text{Ba}(\text{OH})_2$  1 ml of a 25 per cent solution of barium acetate was added, and the barium salt of IGP precipitated by the addition of 4 volumes of alcohol and 2 volumes of acetone The precipitated barium salt was washed twice with acetone

and dried with an on a Buchner funnel. Elemental analyses and evidence for the identity of the isolated product have been presented elsewhere (12). The absorption spectrum of the barium salt of IGP is given in Fig. 4. It is similar to, but not identical with, that of indole.

*Conversion of Indole Glycerol Phosphate to Indole*—It has been already noted (see Fig. 2) that addition of Fraction B to a mixture previously incubated with Fraction A results in the production of indole. Incubation of isolated IGP with Fraction B also results in the production of indole,

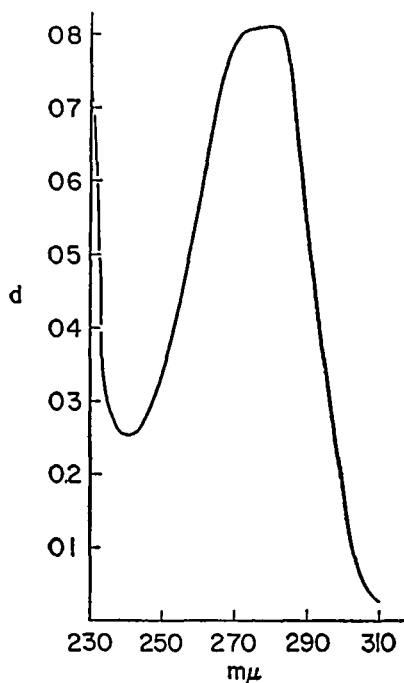


FIG. 4. Absorption spectrum of the barium salt of indole-3-glycerol phosphate (200  $\gamma$  per 3 ml. in water).

with a yield of 80 to 95 per cent of theoretical. In one large scale experiment, the indole formed from IGP was isolated as the picrate to establish its identity unequivocally. The isolated picrate had the same melting point as an authentic sample of indole picrate (174–175°), and the mixed melting point was also the same.

Enzymatic hydrolysis of IGP would be expected to yield equimolar amounts of indole and 3-phosphoglyceraldehyde. Tests performed to detect triose phosphate during the conversion of IGP to indole indicated the presence of a compound which gives a 2,4-dinitrophenyl osazone (12) with an absorption spectrum identical with that of the 2,4-dinitrophenyl osazone formed by the triose phosphates (21). Alkali-labile phosphate also appears, thus further suggesting that triose phosphate is a reaction

product The results of two experiments in which indole formation and alkali-labile phosphate formation were compared are presented in Table II It can be seen that in the absence of KCN approximately 40 per cent of the phosphate expected, on the basis of the indole formed, appears as alkali-labile phosphate, while in the presence of KCN there is fair agreement between indole formation and alkali-labile phosphate formation Additional tests with Fraction B showed that this fraction contains a CN sensitive phosphatase which is active on triose phosphate, generated from hexose diphosphate with aldolase

TABLE II

*Indole and Alkali-Labile Phosphate Formation from Indole Glycerol Phosphate*

Experiment No	Without KCN				With KCN			
	P <sub>i</sub>	P <sub>i</sub> after alkali	Alkali-labile P	Indole formed	P <sub>i</sub>	P <sub>i</sub> after alkali	Alkali-labile P	Indole formed
	$\mu\text{mole}$	$\mu\text{mole}$	$\mu\text{mole}$	$\mu\text{mole}$	$\mu\text{mole}$	$\mu\text{moles}$	$\mu\text{mole}$	$\mu\text{mole}$
1	0.48	0.8	0.32	0.78	0.14	0.9	0.76	1.0
2	0.55	0.82	0.27	0.81	0.14	1.1	0.96	0.99

Each tube contained 1.5  $\mu\text{moles}$  of IGP, 0.25 ml of Fraction B, and 0.1 ml of 0.5 M Tris buffer at pH 7.8 in a final volume of 1 ml As indicated, 0.2 ml of a 0.2 M neutralized solution of KCN was also present Incubation was at 37° for 25 minutes 0.1 ml of the incubation mixture was removed for indole assay and 0.4 ml of 10 per cent trichloroacetic acid was added to the remainder After removal of the precipitate, 0.5 ml aliquots of the supernatant solution were analyzed for P<sub>i</sub> and for alkali labile phosphate (the aliquot was mixed with an equal volume of 2 N KOH and neutralized after 20 minutes)

Indole-3-glycerol, prepared by treating IGP with intestinal phosphatase, is not converted to indole by Fraction B Maximal activity of Fraction B is obtained at about pH 6 The name "indole glycerol phosphate hydrolyase" is proposed for the enzyme which converts IGP to indole

*Indole Glycerol Phosphate Formation from Indole and Triose Phosphate*—In view of the fact that the enzymatic hydrolysis of IGP rarely went to completion, an attempt was made to determine whether the reaction was reversible Fraction B was incubated with indole, aldolase, and hexose diphosphate, and the reaction mixture was analyzed for indole disappearance and IGP formation The results of a typical experiment are shown in Table III It can be seen that Fraction B catalyzed the utilization of indole and the formation of IGP To determine whether the reaction product was indeed IGP, an aliquot from a similar experiment was chromatographed on paper with the developing solvent employed previously, supplemented with 1 ml of 1 N NH<sub>4</sub>OH per 100 ml A spot reacting with

ferric chloride appeared at the same  $R_F$  as IGP when the sample was chromatographed alone or with added IGP. In addition, when the material present in a duplicate unspayed spot was eluted and treated with metaperiodate, indole-3-aldehyde, identified by its characteristic absorption spectrum, was formed. It appears, therefore, that Fraction B catalyzes both the conversion of IGP to indole and 3-phosphoglyceraldehyde and the reverse reaction, the formation of IGP from indole and triose phosphate. Whether these two activities are due to the same enzyme or to two separate enzymes remains to be determined.

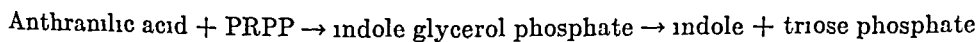
TABLE III  
*Indole Glycerol Phosphate Formation from Indole and Triose Phosphate*

	Indole disappearing	IGP formed
	$\mu\text{mole}$	$\mu\text{mole}$
Complete system	0.25	0.19
Minus Fraction B	0	0
“ aldolase	0	
“ hexose diphosphate	0	
“ indole		0

Each tube contained 0.6  $\mu\text{mole}$  of indole, 15  $\mu\text{moles}$  of hexose diphosphate, 0.2 ml of aldolase, 0.24 ml of Fraction B, and 0.2 ml of 0.5 M phosphate buffer at pH 7.8 in a final volume of 1.4 ml. Incubation was at 37° for 30 minutes. The residual indole was extracted with toluene, and indole assays were performed on aliquots of the toluene layer. An aliquot from the aqueous layer was assayed for IGP by metaperiodate oxidation.

#### DISCUSSION

The data presented in this paper indicate the following mechanism of indole biosynthesis in *E. coli*:



This scheme is also supported by the results of enzymatic and metabolite accumulation investigations.<sup>2</sup> These studies have shown that tryptophan auxotrophs of *E. coli*, blocked in the conversion of anthranilic acid to indole, fall into two groups, according to the content of enzymes involved in the above reactions. One group specifically lacks one or more of the enzymes involved in IGP formation from anthranilic acid, while the second group contains these enzymes but lacks IGP hydrolase. The inability of these mutants to synthesize tryptophan associated with the absence of specific enzymes would appear to be strong evidence for the view that the reactions being considered represent the principal pathway of tryptophan synthesis.

<sup>2</sup> C. Yanofsky, unpublished data.

in *E coli* Data on metabolite accumulation also lend support to this view. Mutants of the group lacking IGP hydrolase, those which might be expected to accumulate IGP, accumulate a compound which appears to be IG (the accumulated compound has the same  $R_F$  in several solvent systems as IG formed from IGP by treatment with intestinal phosphatase). An unidentified compound with properties similar to IG has previously been reported to be accumulated by certain tryptophan auxotrophs of *E coli* (22) and of *Salmonella typhimurium* (23). In addition, the group of mu-

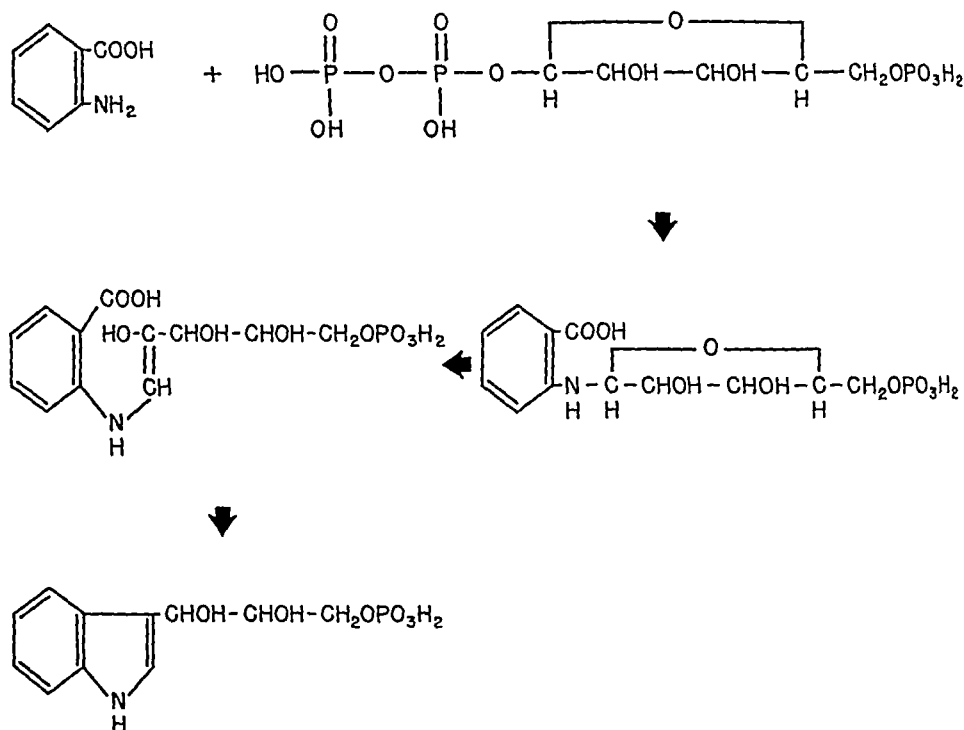


FIG 5 Hypothetical scheme of IGP formation from anthranilic acid

tants unable to form IGP from anthranilic acid accumulate anthranilic acid. Thus accumulation by both groups of mutants is consistent with the postulated scheme of indole biosynthesis.

In growth tests performed with IGP and IG, neither compound was found capable of supporting the growth of auxotrophs which respond to anthranilic acid. This observation could be explained by assuming that *E coli* is impermeable to IGP and that IG, on the other hand, cannot be rephosphorylated. In this connection, it may be noted that intermediates in histidine synthesis, imidazole glycerol and imidazole glycerol phosphate, are also incapable of supporting the growth of histidine mutants which might be expected to respond to one or both (24).

The data presented in this paper provide no clues as to the detailed

mechanism by which anthranilic acid is converted to IGP. By analogy with other reactions in which PRPP participates (25, 26), it seems likely that the first step would involve the formation of the ribotide of anthranilic acid (see Fig. 5, for hypothetical scheme of IGP formation). This compound could be converted by a reaction similar to the Amadori rearrangement (27) to a 1-deoxy, 2-keto intermediate which, in the enol form, would have both the double bond of the pyrrole ring of indole and a hydroxyl group on the 2nd carbon atom of the side chain. The hydroxyl group would then be in position for ring closure with the carbon atom of the benzene ring to which the carboxyl group is attached. Finally, either ring closure followed by decarboxylation or decarboxylation followed by ring closure would give IGP.

In view of the present evidence for the participation of IGP as an intermediate in tryptophan synthesis, it is perhaps surprising that in the synthesis of this amino acid the 3-carbon side chain of IGP is removed and replaced from serine. IGP could conceivably give rise to tryptophan more directly by the sequence of reactions which appear to be involved in histidine synthesis (24), namely, the conversion of a glycerol phosphate side chain to an alanine side chain. In view of the data presented in this paper which suggest that the conversion of IGP to indole is an essential step in tryptophan synthesis, it seems unlikely that this pathway is operative in *E. coli*. It remains to be determined whether it is employed by other microorganisms.

#### SUMMARY

The mechanism of the enzymatic conversion of anthranilic acid to indole in *Escherichia coli* has been investigated. Ammonium sulfate fractionation of extracts of a tryptophan auxotroph of *E. coli* has provided two separate fractions which catalyze successive reactions in the conversion of anthranilic acid to indole. One fraction catalyzes the formation of indole-3-glycerol phosphate from anthranilic acid and 5-phosphoribosyl-1-pyrophosphate. A procedure for the isolation of IGP has been developed. The second fraction converts indole glycerol phosphate to indole and triose phosphate and also catalyzes the reverse reaction, the formation of indole glycerol phosphate from triose phosphate and indole. The significance of these reactions in the biosyntheses of indole and tryptophan is discussed.

The author is indebted to Dr. D. Goldthwait for supplying the labeled and unlabeled samples of 5-phosphoribosyl-1-pyrophosphate and for many interesting and helpful discussions during the course of this work. It is also a pleasure to acknowledge the valuable technical assistance of Mrs. N. Deyczakowsky.



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# ESSENTIAL ROLE OF HISTIDINE PEPTIDES IN TETANUS TOXIN PRODUCTION\*

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A number of papers from this laboratory have traced the development of a medium for the production of highly potent tetanus toxin, the basis of which is a pancreatic digest of casein (1-3). Considerable work has been done in an effort to identify those components of the digest essential for toxin production, and it has been shown that at least some of these materials are peptides (4, 5). In the present communication, experiments are reported which resulted in the identification of one such group of peptides.

## EXPERIMENTAL

*Medium and Methods*—Procedures for maintaining the strain (*Clostridium tetani*, Harvard), preparation and inoculation of the test medium, and assay of the toxin have been described elsewhere (2, 5).

*Fractionation of Digest*—The general scheme for the initial fractionation of the pancreatic digest has been previously described (5), and is outlined in Fig 1. The digest is first separated, by the use of reversible resin columns, into an acidic, a neutral, and a basic fraction. All three must be included in the medium in order to obtain toxin, and each contains at least one substance necessary for toxin production, which is destroyed by acid hydrolysis.

The basic fraction, making up about 20 per cent of the total solids of the digest, appeared to be the simplest one with which to attempt further separation. This fraction was shown by paper chromatography to contain considerable quantities of free arginine, histidine, and lysine, as well as peptides of each, and lesser amounts of other amino acids. Silver precipitation following the general procedure of Vickery and Leavenworth (6) effected a reasonably satisfactory separation of this basic fraction into three subfractions: (a) a precipitate at pH 7.6 containing free histidine and histidine peptides, (b) a further precipitate at a pH beyond 10 containing free arginine and arginine peptides, and (c) a filtrate containing free lysine and lysine.

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† Deceased, February 16, 1954.

peptides These three subfractions could together replace the original basic fraction in the toxin medium The arginine and lysine fractions were

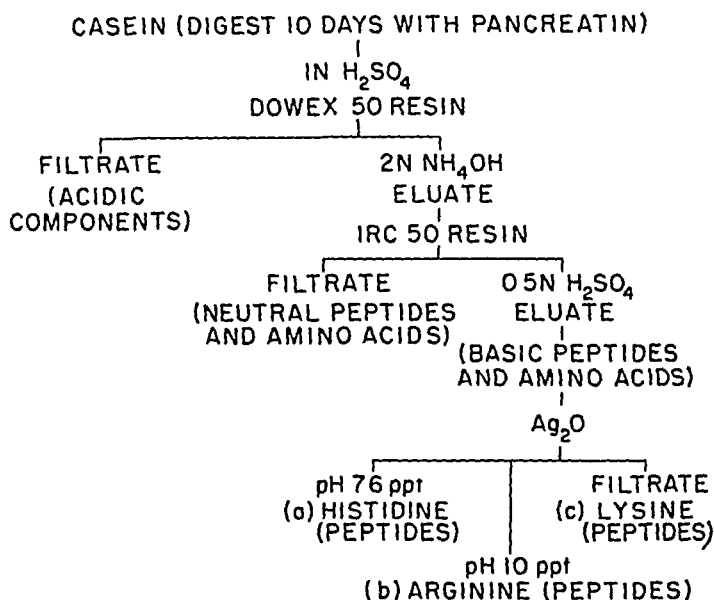


FIG 1 Initial fractionation of pancreatic digest on resin columns

TABLE I  
*Effect of Replacing Basic Fraction with Free Amino Acids*

Addition to basal medium*						Toxin titer
						<i>Lf per ml</i>
Complete pancreatic digest						100
Acid fraction + neutral fraction						Nil
"	"	+	"	"	+ complete basic fraction	75
"	"	+	"	"	+ fractions (a) + (b) + (c)†	70
"	"	+	"	"	+ fraction (a) + L-arginine + L-lysine	70
"	"	+	"	"	+ none + " + "	Nil
"	"	+	"	"	+ L-histidine + L-arginine + L-lysine	"

\* For the constitution of the basal medium, see Mueller and Miller (2)

† See Fig 1

replaceable by free arginine and free lysine, respectively, but free histidine would not substitute for the histidine fraction (a), as is shown in Table I

*Histidine Fraction*—Paper chromatography before and after hydrolysis showed that there were a number of amino acids and peptides in this fraction. Peptides of aspartic and glutamic acids which would form silver salts more or less insoluble at neutral reaction are undoubtedly present. Further separation was attempted and partial success achieved by the use of heavy

sheets of filter paper (Schleicher and Schuell, No 470A) on which a considerable amount (100 mg) of material could be chromatographed. These sheets were fitted at the top with a valve of Whatman No 1 paper, which slows down the rate of flow and consequently improves the separation (7). The histidine fraction, concentrated to a syrup, was applied as a band across the top of the paper. The sheets were developed by the descending technique, a drop of phenol red serving as a convenient marker. The most useful solvent systems were 5 per cent formic acid in secondary butanol and 10 per cent aqueous concentrated  $\text{NH}_4\text{OH}$  in secondary butanol, both half saturated with water. At the completion of the run, the paper was thoroughly dried and a number of narrow longitudinal strips were cut from the edge or the middle of the sheet. One such strip was sprayed with ninhydrin, and the others were used to locate histidine and other amino acids. This was done by laying a strip on the surface of the agar in a large Pyrex baking dish containing minimal medium agar seeded with a suitable auxotrophic mutant of *Escherichia coli*. Free histidine and peptides containing histidine were readily located on a plate seeded with a histidine-requiring mutant, by the areas of growth around that part of the paper to which the histidine or its peptides had migrated. A tracing of these areas on transparent paper made it possible to cut the original chromatographed sheet into appropriate sections. Each section was extracted with water, followed by 0.01 N  $\text{H}_2\text{SO}_4$ . The sulfate was then removed with  $\text{Ba}(\text{OH})_2$  and the extracts concentrated *in vacuo*. Toxin tests indicated certain of these extracts to be effective in replacing the histidine fraction. These active fractions were further examined, both before and after hydrolysis, by paper chromatography, by the use of auxinographic mutant plates, and by electrophoresis with use of Durrum's paper strip procedure (8). Histidine, in combination with many different amino acids, was found in all fractions which were active. Three peptides containing histidine and isoleucine and two containing histidine and lysine were identified. The general distribution of toxin-producing components in the histidine fraction was determined by this method, but the amount of material which could be thus separated was insufficient for further testing and analysis. Other methods of fractionation were therefore investigated.

*Separation of Histidine Fraction on Resin Columns*—A satisfactory separation was obtained by the use of resin columns and elution with volatile buffers, which could then be removed by sublimation, according to the method of Hirs, Moore, and Stein (9). Dowex 50 (250 to 300 mesh, 4 per cent cross linkage) was prepared according to their procedure and suspended in 0.2 M ammonium acetate buffer of pH 5.46. The histidine fraction was concentrated *in vacuo* to a syrup, taken up in a few ml of the 0.2 M ammonium acetate buffer, acidified with acetic acid to pH 2.0, and ap-

plied to the column Three ammonium acetate buffers were used successively for elution, all prepared with glass-distilled water, redistilled acetic acid, and ammonia freshly distilled into water The rate of flow and the volume collected per tube varied with the size of the column

*Analysis of Effluent Fractions*—Fractionation was followed in three ways (1) A loopful from each tube was placed on a large glass slide and, when dry, observed for the appearance, nature, and amount of crystalline material (2) By applying a loopful of fluid from each tube to a sheet of Whatman No 1 filter paper and chromatographing for 3 or 4 hours in one or other of the solvent systems mentioned above, the ninhydrin-positive materials were located and grouped (3) The most useful method of following the separation was to examine the effect of the fluid in each tube on the growth of auxotrophic mutants of *E coli* Small disks of thick filter paper were dipped into each tube and laid on the surface of the agar in plates prepared with minimal agar seeded with the organism Within a few hours, or in some cases overnight, areas of growth around certain of the disks were clearly visible The diameters of the growth areas, which are roughly proportional to the amount of amino acid present, were measured The results obtained from one such column by this technique and with use of four mutants requiring, respectively, histidine, lysine, isoleucine, and arginine, are plotted in Fig 2 This was an early experimental column on which about 60 mg of histidine fraction were separated Free histidine was located in tubes 121 to 139, while the other histidine peaks represent one or more peptides containing histidine Isoleucine is superimposed in part on the histidine line, as it is also present in some of the histidine peptides Valine and phenylalanine emerge early, and are not shown The solid line at the end represents histidine and lysine eluted by the 2 M  $\text{NH}_4\text{OH}$  Larger columns were later used on which more material could be fractionated, and a somewhat better separation of the histidine peaks was obtained

*Removal of Buffers*—The fractions making up each histidine peak were pooled and evaporated *in vacuo* to approximately 0.1 volume (10) and the concentrate was neutralized with 1 M  $\text{NH}_4\text{OH}$ , diluted ten times with water, and again evaporated, this time to a thick syrup The flask containing the syrup was attached to an oil pump fitted with a cold trap, and rotated by hand while evacuation continued, until a fine semicrystalline film was deposited on the sides of the flask Crystallization could be induced by letting a little air in through the stopcock or by seeding with a few crystals of ammonium acetate Once crystals had formed, the flask was left on the pump until thoroughly dry The buffer salt was then sublimed with use of a water-cooled finger type condenser, the flask being immersed in a water bath at 30–40° When all of the ammonium acetate had been removed, the residue was dissolved in water

*Naturally Occurring Peptides*—In this way a number of fractions were obtained which were reasonably pure, which were active, and all of which were found to contain histidine in peptide form. None of these fractions was obtained analytically pure, but they were relatively free the one from the other, and each, if used in adequate quantity (3 to 5 mg in 5 ml of medium), was capable of replacing the entire histidine fraction in the medium used for toxin production, although free histidine was without effect. Considerable information regarding the other amino acids present in these active fractions was obtained. Examination subsequent to hydrolysis showed the presence of one or more of the following amino acids in addition

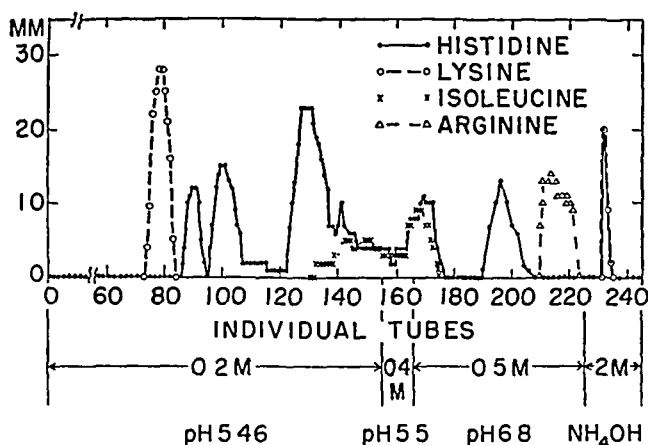


FIG 2 Separation of 60 mg of the histidine fraction on a 2 × 40 cm column of Dowex 50 × 4. Ordinate, diameter of growth around filter paper disks on mutant plates. Abscissa, the number of tubes. Effluent collected in 4 ml fractions, flow rate about 10 ml per hour. The pH and molarity of the ammonium acetate buffers are indicated.

to histidine: glutamic acid, lysine, alanine, glycine, isoleucine, leucine, methionine, arginine, proline, and valine. Some of these were undoubtedly present as impurities. There were none common to all of the active fractions, and there was no evidence for a specific linkage.

*Synthetic Histidine Peptides*<sup>1</sup>—A number of synthetic histidine peptides were tested and were found to be effective in replacing the histidine peptide

<sup>1</sup> Through the kindness of Dr. du Vigneaud of the Cornell Medical College, we were able to test six synthetic histidine peptides prepared in his laboratory. These were L-carnosine, β-L-aspartyl-L-histidine, glycyl-L-histidine, L-α-amino-n-butyl-L-histidine, L-alanyl-L-histidine, and D-alanyl-L-histidine. Anserine, 1-methyl-DL-histidine, and 3-methyl-DL-histidine were kindly sent to us by Dr. D. Wright Wilson of the University of Pennsylvania. We are indebted to Dr. R. W. Holley of the New York State Experimental Station for the L-histidyl-L-alanine. Acetyl-L-histidine was prepared in this laboratory by Dr. Harry Gooder by the procedures described (11, 12).

in the naturally occurring material Table II lists these in the approximate order of activity It is of interest that neither anserine, 1-methyl-DL-histidine, nor 3-methyl-DL-histidine had any effect on toxin production when tested under conditions in which carnosine and other peptides of histidine were effective Anserine, when added in addition to carnosine, did not inhibit the effect of the carnosine

TABLE II  
*Comparative Activities of Histidine Peptides in Toxin  
Production by C. tetani*

Compound*	Toxin titer
Glycyl-L-histidine	Maximal†
L- $\alpha$ -Amino- <i>n</i> -butyryl-L-histidine	"
$\beta$ -L-Aspartyl-L-histidine	"
Acetyl histidine	"
L-Carnosine	"
L-Alanyl-L-histidine	90% maximal‡
D-Alanyl-L-histidine	80% "
L-Histidyl-L-histidine	60% "
L-Histidyl-L-alanine	60% "
$\alpha$ -L-Glutamyl-L-histidine	50% "
L-Leucyl-L-histidine	40% "
Anserine	Inactive
1-Methyl-DL-histidine	"
3-Methyl-DL-histidine	"
L-Histidine	"

\* Peptides in approximate order of activity

† "Maximal" titer was taken as that routinely obtained on a medium containing the whole pancreatic digest of casein The actual amount of peptide necessary to produce this varied from 0.05 mg of glycyl-L-histidine to over 5 mg of carnosine

‡ This and the following compounds never achieved "maximal" titer, even when amounts up to 10 mg were used

With the still relatively crude base, which contained the acidic and neutral fractions of the casein digest, it has not been possible to carry out completely comparative quantitative assays It could be determined, however, that toxin is produced in response to a number of histidine dipeptides, and that there are quantitative differences in their effect The titers of toxin obtained when various amounts of four peptides are added to the basal medium are plotted in Fig. 3 The most active peptides tested were glycyl-L-histidine and L- $\alpha$ -amino-*n*-butyryl-L-histidine Acetylhistidine and carnosine are effective, but high yields of toxin are obtained with these substances only when large amounts are used

*Glycyl-L-histidine*—This peptide was the most active of those tested

The titers of toxin obtained on the addition of increasing amounts of this substance to an otherwise complete medium are seen in Table III. The

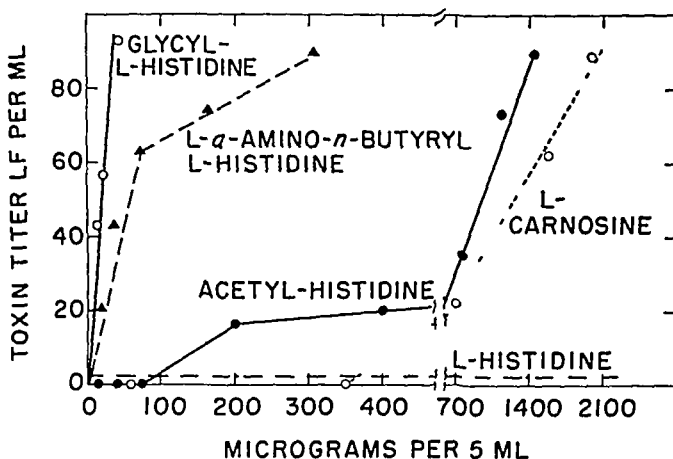


FIG 3 The comparative effect of histidine peptides on toxin production by *C tetani*. The basal medium contains 1.25 mg of L-histidine per 5 ml.

TABLE III

*Effect of Glycyl-L-histidine on Tetanus Toxin Production*

Addition to medium*	Amount added	Toxin titer
	mg per 5 ml	Lf per ml
Nothing		Nil
Glycyl-L-histidine	0.1	40
"	0.25	110
"	0.5	80
"	1.0	110
"	1.5	130
"	2.0	130
"	2.5	130
Pancreatic digest	115	135
" "	115	140

\* Basal medium (2), to which the acidic and the neutral fractions of the pancreatic digest have been added. The three subfractions of the basic fraction have been replaced by L-histidine, L-arginine, and L-lysine.

addition of as little as 1.5 mg to 5 ml of medium gave essentially as high a toxin titer as could be obtained with the complete pancreatic digest.

*Preparation of Peptides*—With the generous help and cooperation of Dr Max Bovarnick and Dr E. Borek of the Veterans Administration Hospital, Brooklyn, in whose laboratories one of us (J. H. M.) carried out some of



these syntheses, a number of peptides were prepared, including  $\alpha$ -L-glutamyl-L-histidine, leucyl-L-histidine, and histidyl-L-histidine

Glycyl-L-histidine was prepared in this laboratory from chloroacetyl-L-histidine by the general method of Rao *et al* for other glycyl peptides (13). It was found that the chloroacetyl intermediate could be purified and crystallized by passing the crude reaction mixture through a triple column of Dowex 2, and by displacement with dilute HCl, according to the technique of Partridge and Brimley (14). After amination, the peptide was separated from by-products by fractionation on triple columns of Dowex 50, eluting with dilute ammonium hydroxide. Fractions containing combined histidine were pooled and the glycyl-L-histidine was crystallized.

Carnosine was prepared synthetically, and was also obtained from beef skeletal muscle by chromatography on Dowex 50. Carnosine, and anserine if present, could be detected by auxinographic methods with two mutants of *E. coli*, the one responding to histidine or carnosine but not to anserine or methylhistidine, the second responding to  $\beta$ -alanine, carnosine, or anserine. The carnosine fraction obtained was further purified on Amberlite IR-112 with use of ammonium acetate buffers by the method of Hirs *et al* (9). Precipitation of the appropriate fraction with silver produced about 0.6 gm of crystalline carnosine from 1 kilo of skeletal muscle.

A small amount of beef heart infusion is included in the basal medium, and it at first seemed that this should rule out the need for carnosine, the utilization of which was therefore somewhat puzzling. However, reports in the literature (15) indicate that 100 gm of beef heart contain only 4 or 5 mg of carnosine, and, since less than the equivalent of 0.2 gm of fresh tissue is added to 5 ml of medium, this would supply only about 0.01 mg of carnosine and would be ineffective as a source of this peptide. As a further confirmation of the relatively small amount of carnosine in beef heart, attempts to prepare it from this tissue by the method used for obtaining it from beef skeletal muscle were unsuccessful.

*Peptides in Acidic and Neutral Fractions*—There is evidence that the remaining unknown components in the acidic and neutral fractions are also peptide in nature. As has been reported (5), the active material in the acidic fraction appears to be a peptide or peptides containing glutamic acid, isoleucine, and serine, which is combined with the phosphorus.

The neutral fraction is the largest and most complex. The majority of the active fractions obtained by separation on resin columns show on hydrolysis the presence of both lysine and glutamic acid, with a wide variety of other amino acids. Peptides of glutamic acid or glutamine seem a definite possibility, since glutamic acid, although not present as such in the pancreatic digest, is essential for growth of the organism, yet if the L-amino acid is added to the complete medium, the production of toxin is inhibited.

None of the peptides available to us has proved effective in the amounts and combinations tried <sup>2</sup>

The fact that the active material is widely distributed throughout this fraction may indicate activity with several peptides, each containing a common specific amino acid. It does not, however, exclude the possibility that concomitant presence of two kinds of peptides is essential, nor the possibility that the essential materials are not peptide in nature.

#### DISCUSSION

Under our experimental conditions, toxin does not appear in the medium in large amount, readily detectable by flocculation, until about the 3rd day of growth, at which time the culture begins to autolyze. Autolysis is complete by the 4th or 5th day, when the toxin is harvested. If for some reason autolysis does not take place, and it is possible by various methods to prevent this, only a small amount of toxin will be found in the medium. Little is known of the relationship between toxin synthesis and general protoplasmic synthesis. That tetanus toxin is formed and can be demonstrated *within* the cell has been shown by Raynaud (16) and confirmed by us. The striking feature of toxin formation is that it is not directly correlated with the general growth processes, the omission of one essential material (histidine peptides) prevents the production of toxin by the cell, although growth occurs.

The effect of peptides on the growth of microorganisms has been rather extensively studied, and a number of suggestions have been offered to account for the increased activity of peptides as compared with that of the free amino acids. The three most common explanations for the superior effect of peptides over the free amino acids have been recently summarized by Kihara and Snell (17). They are (a) that the free amino acid is rapidly degraded by certain organisms, thereby resulting in a deficiency, (b) that some cells appear to be more permeable to peptides than to the free amino acids, and (c) that certain substances in the medium may inhibit the assimilation of the free acid by the cell. The present situation is unique in that free histidine is utilized by the organism for growth, but even in large amounts it cannot replace the peptide in the formation of toxin. For the formation of toxin by the cell, the histidine must be supplied in peptide form. Moreover, from the observation that acetylhistidine can be uti-

<sup>2</sup> Through the kindness of Dr. Fruton of Yale University, we were able to test the following glutamic acid peptides: glycyl-L-glutamic acid,  $\alpha$ -L-glutamylglycylglycine,  $\alpha$ -L-glutamyl-L-tyrosine, and glycylglycyl- $\alpha$ -glutamylglycine. Glutamyl-L-histidine was prepared in this laboratory. From the late Dr. Erwin Brand of New York University, we received samples of the following lysine peptides: H-Lys-Gly-OH-HCl, H-Glu-OH-L-Lys-OH, H-Gly-Lys-Gly-OH-HCl, H-Lys-Lys-Lys-OH-3HCl, H-Ala-Lys-Ala-Ala-OH-HCl, and H-Ala-Lys-Ala-Ala-Ala-OH-HCl.

lized and the further observations that anserine and the methylhistidines are not effective, it appears that the essential requirement for the formation of toxin is the structural specificity of histidine in peptide linkage

Histidine peptides are hydrolyzed when they are incubated with washed cells of *C. tetani* grown on toxin-producing medium. Further studies on the function of this enzyme in relation to the peptide requirement for toxin production are now in progress.

#### SUMMARY

A pancreatic digest of casein, the key material in a defined medium for the production of tetanus toxin, has been separated by means of reversible resin columns into three main fractions: (1) an acidic fraction, (2) a neutral fraction, and (3) a basic fraction. The participation of the basic fraction in the production of toxin has now been elucidated. By means of silver precipitation it has been separated into three subfractions, free arginine and lysine will substitute for the corresponding two subfractions, and the essential component in the histidine fraction has been identified as histidine in peptide linkage. Synthetic histidine peptides can be successfully substituted for the naturally occurring material, and differences in their effect on toxin production have been observed. Glycyl-L-histidine and  $\alpha$ -amino *n*-butyryl-L-histidine were the most active of those tested. Carnosine or acetylhistidine will substitute, but good toxin is produced only when large amounts of these substances are used. Free L-histidine is without effect on toxin production but will support growth of the organism. Preliminary experiments on the remaining unknown components in the acidic and neutral fractions indicate that they also may be peptide in nature.

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# STUDIES OF THE BIOSYNTHESIS OF OROTIC ACID\*

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It has been established that orotic acid, or a closely related compound, is a precursor of the pyrimidines in nucleic acid (2-9). In studying the synthesis of orotic acid, Lieberman and Kornberg (10) have demonstrated the following reactions in a cell-free extract of *Zymobacterium oroticum*

Ureidosuccinic acid (US)<sup>1</sup>  $\rightleftharpoons$  dihydroorotic acid (DHO)  $\rightleftharpoons$  orotic acid (OA)

US was shown by Reichard and Lagerkvist (11) to be a direct precursor of OA in rat liver slices, and the conversion of US to OA via DHO in rat liver homogenates was established by Cooper and Wilson (12).

The main purpose of this paper is to present observations on the ability of cell fractions of rat liver to convert US to OA.

## Methods

*Fractionation of Rat Liver Homogenates*—The procedure of Hogeboom *et al.* (13) was used. Usually two rats of the Wistar strain were killed, and 15 to 20 gm. of liver were obtained. The percentage recovery of each fraction and the homogeneity of the preparations were similar to those reported (13).

DL-Ureidosuccinic Acid-Ureido-C<sup>14</sup> was synthesized by the method of Nyc and Mitchell (14).

*Isolation and Purification of Pyrimidines*—After the incubation, the proteins were precipitated with an equal volume of 10 per cent trichloroacetic acid (TCA), and the residue was removed by centrifugation and washed

\* The data in this paper have been taken from a thesis presented by Ray Wu to the Graduate School of Arts and Sciences of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy. An abstract of a preliminary report of this work has appeared (1). Aided by a grant from the American Cancer Society, administered by the Committee on Growth of the National Research Council, and by a grant from the Atomic Energy Commission.

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<sup>1</sup> The following abbreviations are used in the present paper: AD, alcohol dehydrogenase; ASU, acid-soluble uracil; ATP, adenosine triphosphate; CoA, coenzyme A; DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; DHO, dihydroorotic acid; FDP, fructose-1,6-diphosphate; OA, orotic acid; TCA, trichloroacetic acid; TPN, triphosphopyridine nucleotide; US, ureidosuccinic acid.

with 10 ml of 5 per cent TCA. The combined supernatant fluid was put through a charcoal column (Dai-co G-60, 60 to 100 mesh, a  $3 \times 1$  cm column gave a flow rate of around 4 ml per minute with air pressure). After being washed with 200 ml of water, the pyrimidines adsorbed on the column were essentially free from TCA, buffer salts, soluble proteins, and the unchanged radioactive US. The pyrimidines were then eluted with concentrated  $\text{NH}_4\text{OH}$ -ethanol- $\text{H}_2\text{O}$  (10:25:65 by volume), and the eluate was dried and purified by chromatography on Whatman No. 3 paper with butanol-acetic acid-water (4:1:1). The OA band ( $R_F = 0.18$ ) and the uracil band ( $R_F = 0.45$ ) were separately eluted with warm water. Usually, the OA eluted from the paper was free from contaminants, but routinely it was put through a Dowex 1-Cl column to assure its purity. With a  $5 \times 0.7$  cm column, when eluted with 0.008 N HCl, the OA was usually collected between tubes 10 and 14 (10 ml per tube). The uracil eluted from the paper was purified on a Dowex 1-OH column.

Samples of 50 to 300  $\gamma$  were counted in stainless steel planchets with a gas flow counter, sufficient counts being made to reduce the standard error to 5 per cent or less.

### Results

#### *Conversion of US to OA in Rat Liver Homogenates*

Previous work (11, 15) on the conversion of US to OA was carried out with either a bank of OA included in the incubation or with carrier OA added at the end, so that direct comparison of the specific activity in the original substrate with that of the newly synthesized OA could not be made. To overcome this disadvantage, we have incubated US-ureido- $\text{C}^{14}$  with a 2:1 rat liver homogenate and have isolated OA directly. That its specific activity is almost the same (about 96 per cent) as the original US- $\text{C}^{14}$  supports the hypothesis that OA is on the direct path of uridylic acid synthesis from US.

#### *Conversion of US to OA in Fractionated Liver Homogenates*

*Ability of Cell Fractions to Synthesize OA from US*—Rat liver homogenates, fractionated in 0.25 M sucrose, gave results (Table I) to indicate that the nuclear, mitochondrial, and microsomal fractions were almost equally active in converting US to OA. The supernatant fraction was completely inactive in this regard.

*Ability of Cell Fractions to Synthesize Acid-Soluble Uracil from US and OA*—A similar set of experiments was carried out to examine the ability of individual cell fractions and the reconstituted homogenate to convert US and OA to acid-soluble uracil. Uracil was isolated from the incubation mixture at the end of the experiment. The finding of uracil rather than

uridylic acid is probably due to the action of degradation enzymes on the acid-soluble uridylic acid under the experimental conditions employed

TABLE I  
*Conversion of US to OA in Cell Fractions*

Each flask contained 5  $\mu$ moles of US-ureido-C<sup>14</sup> (50,000 c p m per  $\mu$ mole), 10  $\mu$ moles of DPN, 10  $\mu$ moles of ATP, and 40  $\mu$ moles of fructose-1,6-diphosphate in 15 ml of phosphate buffer (14) at pH 7.4. All the flasks were incubated at 25° for 4 hours with continuous gassing (95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub>). 10  $\mu$ moles of carrier OA were added at the end of the incubation.

Fraction	Specific activity of OA	N	Specific activity of OA per mg N	OA synthesis per mg N*
	c p m per $\mu$ mole	mg	c p m per $\mu$ mole	$\mu$ mole
Nuclear	4750	50	95	0.0190
Mitochondrial	3400	57	60	0.0120
Microsomal	2460	30	82	0.0164
Supernatant	9	57	0	0
Reconstituted homogenate	3680	70	53	0.0106

\* Micromoles of OA synthesis per mg of N = (specific activity of OA per mg N  $\times$  10)/50,000

TABLE II  
*Synthesis of Acid-Soluble Uracil from US and OA*

Fraction	Acid-soluble uracil* from US		Acid soluble uracil† from OA
	c p m per $\mu$ mole	c p m per $\mu$ mole per mg N	c p m per $\mu$ mole per mg N
Nuclear	23	0	0
Mitochondrial	0	0	0
Microsomal	12	0	3
Supernatant	0	0	21
Reconstituted homogenate	2700	33	

\* The experimental conditions were the same as those given in Table I, except that 5  $\mu$ moles of uracil were added as carrier at the end of the incubation.

† Each flask contained 5  $\mu$ moles of OA-2-C<sup>14</sup> (41,000 c p m per  $\mu$ mole) in phosphate buffer containing DPN, ATP, and FDP, as given in Table I. The flasks were incubated at 32° for 50 minutes with continuous gassing. The enzyme preparation containing about 6 mg of N was used in each experiment. At the end of the incubation, 5  $\mu$ moles of carrier uracil were added.

The data (Table II, second and third columns) indicate that only the reconstituted sample, with supernatant fluid present, synthesized appreciable amounts of uracil from US. The results in the last column (Table II)

show that the particulate fractions of rat liver were unable to convert OA to acid-soluble uracil, while the supernatant fraction was able to carry out this conversion

### *Optimal Conditions for Conversion of US to OA by Particles of Liver Cells*

*Effects of Aging, Freezing, and Versene (Ethylenediaminetetraacetic Acid)*—Aging at room temperature for 3 hours or freezing overnight caused a loss of 20 to 30 per cent of the activity of the nuclear fraction Versene

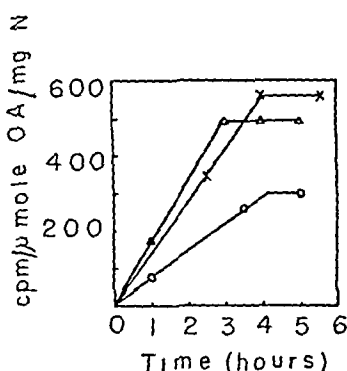


FIG 1

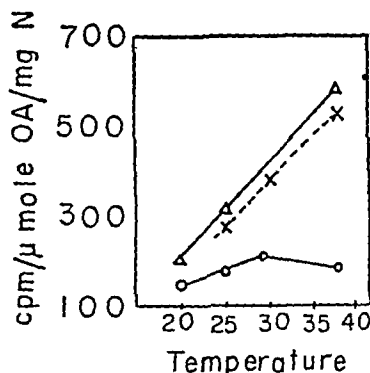


FIG 2

FIG 1 Time curves of OA synthesis Each flask contained 1  $\mu$ mole of US ureido  $C^{14}$ , 0.5  $\mu$ mole of DPN, 3  $\mu$ moles of  $MgCl_2$ , and the enzyme fraction containing about 5 mg of nitrogen They were incubated with phosphate buffer at pH 7.0 at 25° 4  $\mu$ moles of carrier OA were added at the end of the incubation X, nuclear extract, O, mitochondrial extract,  $\Delta$ , microsomal extract

FIG 2 Temperature curves of OA synthesis The conditions were similar to those described in Fig 1 The flasks were incubated for 3 hours

did not reduce the activities of the particles studied This suggests that divalent metal ions are not required by these enzyme systems

*Effect of Time (Fig 1)*—The time curves of orotic acid synthesis were linear until they leveled off between 3 and 4½ hours

*Effect of Temperature (Fig 2)*—For the microsomal fraction, a straight line plot showed an increment of activity with increasing temperature up to 38° For the mitochondrial fraction, however, the optimal temperature was around 28° The curve for the nuclear fraction was similar to that of the microsomal fraction

*Conversion of US to OA in Extracts of Particles, Lysing Agents*—The isolated particles were at first extracted with 10 volumes of water in an attempt to dissolve the enzymes It was found that the water-soluble extract of the mitochondrial fraction had about 35 per cent of the enzyme activity of the intact preparation, but the activities of the nuclear and the microsomal fractions were very low

Among the surface-active agents tested, 1 per cent digitonin<sup>2</sup> in water was found to be the most effective, it released 45 per cent of the activity from the nuclear fraction, 76 per cent from the mitochondrial fraction, and 80 per cent from the microsomal fraction. 1 per cent saponin and 1 per cent sodium dodecyl sulfate (Duponol) gave totally inactive extracts, although the extracts contained considerable amounts of protein. More dilute saponin solutions, deoxycholate, and *n*-butanol all gave inactive extracts. Freezing and thawing, sonic vibration, and treatment in a Wai-

TABLE III

*Effect of Metal Ions on OA Synthesis*

Each flask contained 1  $\mu$ mole of US-ureido-C<sup>14</sup>, 0.5  $\mu$ mole of DPN, and about 1 mg of nitrogen. It was incubated with phosphate buffer, pH 7.0, at 25° for 3 hours. 4  $\mu$ moles of carrier OA were added at the end of the incubation.

Extract	Final concentration of divalent metal ions		Specific activity of OA per mg N
		<i>M</i>	<i>c p m per <math>\mu</math>mole</i>
Nuclear		0	613
	CaCl <sub>2</sub>	0.012	470
	CuSO <sub>4</sub>	0.004	590
	MgCl <sub>2</sub>	0.001	600
Mitochondrial		0	452
	CaCl <sub>2</sub>	0.012	283
	CuSO <sub>4</sub>	0.004	165
	MgCl <sub>2</sub>	0.001	450
Microsomal		0	767
	CaCl <sub>2</sub> during fractionation	0.0018	350
	CaCl <sub>2</sub>	0.012	330
	CuSO <sub>4</sub>	0.004	760
	MgCl <sub>2</sub>	0.001	754

ing blender were also found to be unsatisfactory in dissolving the enzymes for OA synthesis.

*Effect of Calcium, Magnesium, and Cupric Ions (Table III)*—Calcium ions (0.012 *M*) reduced the enzyme activity to 40 to 75 per cent of the original in all three fractions. The enzyme activity in the mitochondrial extract was reduced to one-third of the original in the presence of 0.004 *M* CuSO<sub>4</sub>. Magnesium ions were found to have neither inhibitory nor stimulatory effects under the experimental conditions.

<sup>2</sup> Digitonin (Merck and Company, No. 52223 or 51393) was soluble in water. The digitonin produced at present (No. 50315 or 50334) is insoluble in water, 20 per cent ethanol has to be added to obtain a 1 per cent digitonin solution. 20 per cent ethanol added to the lysed extract was found to have no inhibitory effect on OA synthesis.



*pH Optimum (Fig 3)*—The optimal pH for all three fractions was in the neighborhood of 6.8, with a rather broad maximum from pH 6.4 to 7.2.

*Different Amounts of Enzymes (Fig 4)*—In these experiments 1  $\mu$ mole of US and different amounts of enzyme extracts from the mitochondrial and microsomal fractions were used. The enzyme solutions containing about 4.0 mg of nitrogen appeared to be maximal for 1  $\mu$ mole of US.

*Cofactor Requirements*—For the studies of cofactor requirements, the extract was passed through a  $3 \times 1$  cm charcoal column to remove the free diphosphopyridine nucleotide (DPN), adenosine triphosphate (ATP), and coenzyme A (CoA). The charcoal-treated extract had lost none of

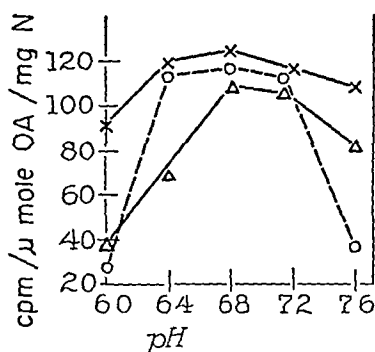


FIG 3

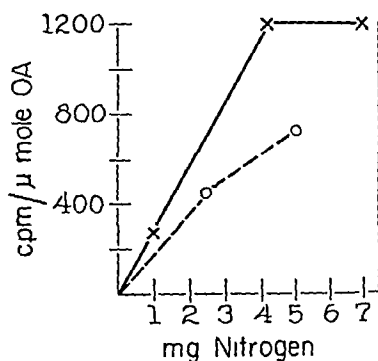


FIG 4

FIG 3 Effect of pH on OA synthesis. The experimental conditions were similar to those described in Table III. X, nuclear extract, O, mitochondrial extract, Δ, microsomal extract.

FIG 4 Synthesis of OA with different amounts of enzymes. The experimental conditions were similar to those described in Table III, except that each flask contained from 0.7 to 7.0 mg of nitrogen. X, mitochondrial extract, O, microsomal extract.

its activity. It contained more than 90 per cent of the original nitrogen content, but the spectrophotometric readings were reduced to between 60 and 75 per cent of those of the untreated extract.

*DPN Requirement*—The addition of DPN to the charcoal-treated preparation gave practically no increase of activity. The microsomal extract was further treated by adding solid ammonium sulfate to 70 per cent saturation. The mixture was centrifuged and the precipitate was dissolved in 20 ml of buffer at pH 7.0. It was dialyzed at 4° against 1 liter of 0.05 M sodium phosphate buffer (pH 7.0) for 6 and for 24 hours. The addition of DPN or triphosphopyridine nucleotide (TPN) to this preparation still showed no consistent stimulatory effect on OA synthesis.

Since the results of the direct stimulation by DPN were uncertain, alcohol dehydrogenase and acetaldehyde were used, not only because alcohol dehydrogenase was reported to be specifically linked to DPN (16) but also

because the equilibrium is in favor of DPN formation. The data in Table IV show that the alcohol dehydrogenase and acetaldehyde produced a 14-fold increase in OA synthesis in the mitochondrial extract and a 7-fold increase in the microsomal extract.

TABLE IV

*Effect of AD on DPN Requirement*

Each flask contained 1  $\mu$ mole of US-ureido-C<sup>14</sup> in 5 ml of phosphate buffer (pH 7.0). The enzyme fraction contained about 1 mg of N per experiment. The mitochondrial extract was incubated at 25° and the microsomal extract at 38° for 3 hours. 4  $\mu$ moles of carrier OA were added at the end of the incubation. 0.1 ml of crystalline yeast alcohol dehydrogenase was used, containing 340,000 units (17).

Charcoal-treated extract	Addition	Specific activity of OA per mg N
		<i>c p m per <math>\mu</math>mole</i>
Mitochondrial	None	266
	25 $\mu$ moles CH <sub>3</sub> CHO, 0.1 ml AD	3760
Microsomal	None	272
	25 $\mu$ moles CH <sub>3</sub> CHO, 0.1 ml AD	1940

TABLE V

*Effect of AD on OA Synthesis in Mitochondrial Extract*

The conditions were the same as those given in Table IV, except that the flasks were incubated at 28° for 3 hours.

Extract	Addition	Actual counts in OA
<i>ml</i>		<i>c p m per <math>\mu</math>mole</i>
1	None	333
1	25 $\mu$ moles CH <sub>3</sub> CHO	321
1	0.1 ml AD	1330
1	CH <sub>3</sub> CHO, AD	1260
2	" "	1820
1	" " 0.5 $\mu$ mole DPN	1240
1	" boiled AD	336
0	" AD	27

To get more detailed information about the effect of alcohol dehydrogenase (AD), data in Table V were obtained. The observation that AD gave the same stimulation without the addition of acetaldehyde probably indicates that there were enough aldehydes present in the extract. No further stimulation upon addition of DPN suggests that there were sufficient amounts of DPN in the extract to support maximal OA synthesis. With 1 ml of extract the rate of OA synthesis was probably limited by the

amount of the necessary enzymes, since more OA was formed with 2 ml of extract

The DPN requirement was also suggested when the incubation was made anaerobic. The enzyme extracts from mitochondria and from microsomes, each with phosphate buffer, were placed in different Thunberg tubes and radioactive US was placed in the bulbs. The tubes were evacuated with an oil pump and refilled with nitrogen. The process was repeated twice. The US was tipped in and the solutions were allowed to digest anaerobically for 3 hours at 25° and 38°. The specific activity of the OA formed was

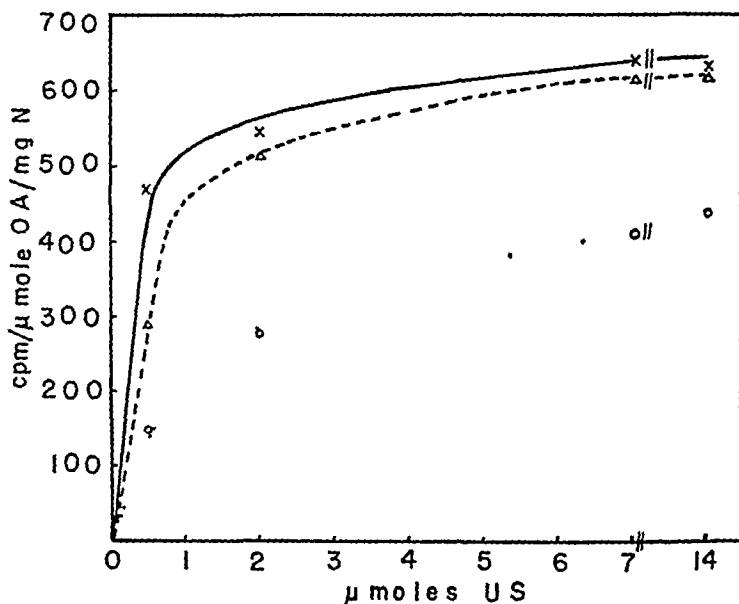


Fig 5 Synthesis of OA with different amounts of substrate. The experimental conditions were similar to those given in Table III. All the flasks were incubated at 32° for 3 hours. The final volume was 8 ml. X, nuclear extract, Δ, mitochondrial extract, O, microsomal extract.

greatly reduced in the anaerobic tubes and was higher if air was permitted to enter when the US was tipped in. The results suggest that DPN and DPNH oxidase may be involved since the synthesis of OA (a) was reduced to about a fourth under anaerobic conditions, and (b) returned toward the control values after air was admitted.

**ATP Requirement**—No stimulation of formation of OA could be demonstrated with the addition of varying amounts of ATP to the charcoal-treated extracts of nuclear, mitochondrial, and microsomal fractions. The addition of hexokinase and glucose, which remove ATP effectively, failed to reduce the incorporation of radioactivity into OA even when hexokinase was added at 0, 50, 100, and 150 minutes during the digestion with a charcoal-treated microsomal extract.

*CoA Requirement*—The addition of varying amounts of CoA incubated for 0.5, 1, and 3 hours showed no stimulation of OA synthesis with the charcoal-treated nuclear, mitochondrial, and microsomal extracts

*Varying Amounts of Substrate*—The rates of formation of OA were determined by using fairly small amounts of crude enzyme extract and different amounts of US. The results are plotted in Fig. 5

#### DISCUSSION

The problem of whether OA is on the direct pathway of pyrimidine nucleotide synthesis has never been settled. Mitchell *et al.* (18) concluded, from genetic evidence, that OA was not a "normal" intermediate in pyrimidine biosynthesis in *Neurospora*. On the other hand, Reichard (19), by working with rat liver slices, favored the suggestion that OA is a "normal" intermediate (20).

We have presented evidence which supports the idea that OA is a "normal" intermediate. (a) ATP is not required for the synthesis of OA from US in extracts of rat liver mitochondria and microsomes. It would seem that ATP would be required if orotidine-5- $\text{PO}_4$  were formed before OA was formed. The conversion of OA to uridylic acid requires ATP (20, 21), as would be expected if OA were converted to orotidine-5'- $\text{PO}_4$  before being changed to uridylic acid. (b) Upon incubating US-uracil- $\text{C}^{14}$  with rat liver homogenate, we have isolated OA with almost undiluted specific activity. The introduction of one or more additional compounds between US and OA makes possible a greater dilution of the final specific activity of OA.

OA may be converted into the uracil of nucleic acid and into acid-soluble uridylic acid and acid-soluble uracil (ASU) in rats (15, 20). Acid-soluble uridylic acid may be incorporated into the pyrimidines of nucleic acid (22). Uracil may be derived from uridylic acid (21), but does not form uridylic acid nor the pyrimidines of nucleic acid (23),<sup>3</sup> therefore the reaction, uridylic acid  $\rightarrow$  uracil, is practically irreversible. Orotic acid is converted into orotidine monophosphate and this reaction occurs before uridylic acid is formed (21).

Accordingly, it seems to us not unreasonable to think that orotic acid is incorporated first into orotidine monophosphate and then into uridylic acid. Uridylic acid may form ASU. It has not been determined whether all of the compounds known to be incorporated into pyrimidines of nucleic acid are formed into nucleosides or nucleotides before they undergo further

<sup>3</sup> Reichard (23) found small amounts of radioactivity incorporated in the nucleic acid pyrimidines when radioactive uracil was injected into rats. However, he recognized that the amount incorporated was such that it might come from  $\text{CO}_2$  formed by the oxidation of some of the uracil.

changes. However, the fact that US may be changed by tissue enzymes into DHO and OA without addition of ATP suggests that the reactions leading to OA formation may occur without nucleoside or nucleotide formation.

The sequence of polynucleotide pyrimidine biosynthesis, as suggested by the work of others and of ourselves, may be postulated as follows: aspartic acid  $\rightarrow$  US  $\rightarrow$  DHO  $\rightarrow$  OA  $\rightarrow$  orotidine-5'-PO<sub>4</sub>  $\rightarrow$  uridylic acid  $\rightarrow$  polynucleotide pyrimidines.

Studies with fractionated rat liver homogenates have revealed that the enzyme systems responsible for the synthesis of OA from US are fairly evenly distributed in all three particulate fractions. The distribution of this system possibly reflects the importance of these two compounds as normal intermediates in pyrimidine biosynthesis. The occurrence of the enzyme systems for OA synthesis in the particulate fractions and those for converting OA to ASU in the supernatant fraction is another example of a complicated metabolic process which is carried out by integrated actions of several cell structures. It is interesting to note that the conversion of US to OA requires no ATP and Mg<sup>++</sup> ions, whereas the conversions of aspartic acid to US (24) and OA to uridylic acid (20) are reported to require both

#### SUMMARY

1 Evidence has been obtained with rat liver homogenates and water soluble extracts to support the theory that orotic acid (OA) is on the direct pathway of pyrimidine nucleotide synthesis.

2 The nuclear, mitochondrial, and microsomal fractions of rat liver were almost equally active in converting ureidosuccinic acid (US) to OA, while the supernatant fraction was totally inactive. On the other hand, the latter was able to convert OA to acid-soluble uracil, while none of the particulate fractions was active in this conversion.

3 The optimal conditions for OA synthesis, including temperature, incubation time, and pH, were determined.

4 The enzyme systems were extracted from particles with 1 per cent digitonin.

5 Diphosphopyridine nucleotide was found to be required for the conversion of US to OA, while no requirements for adenosine triphosphate, coenzyme A, or Mg<sup>++</sup> ions could be demonstrated. Whereas calcium ions inhibited the enzymes from all three fractions, cupric ions inhibited only the mitochondrial enzymes. Versene was found to protect the enzymes.

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# A NEW METABOLIC PATHWAY FOR A SULFONAMIDE GROUP

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The sulfonamide group is generally classified among the more stable functional groupings from a chemical standpoint, and experience has shown this generalization to apply in the animal body as well. Williams states that "the aromatic sulfonamide group appears to resist transformation *in vivo* and is usually excreted as such" (1) <sup>1</sup>

Heterocyclic sulfonamides ( $R-SO_2NH_2$ , where  $R$  = heterocyclic ring) are often less stable to hot acid than the aromatic analogues, being hydrolyzed to the corresponding  $R-OH$  compounds in some cases (2). This report indicates that they may be more susceptible to metabolic degradation as well.

A number of heterocyclic sulfonamides (2) have been found (3) to be powerful inhibitors of carbonic anhydrase *in vitro*. Some of them, such as 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox, acetazolamide), augment the excretion of bicarbonate, sodium, potassium, and water by the dog (4) and by man (5), presumably because of their carbonic anhydrase-inhibitory properties. Others are nearly or totally devoid of activity *in vivo*, one of the most interesting of these being 2-benzothiazolesulfonamide.

This compound, although about twice as active as acetazolamide *in vitro*, produces only a mild increase in bicarbonate output by the dog, even upon massive intravenous dosage (200 mg per kilo). Whereas acetazolamide given in this manner can readily be isolated in unchanged form from the urine to the extent of about 70 per cent of the dose (6), the urine of a dog receiving benzothiazolesulfonamide shows little inhibition of carbonic anhydrase by the assay method *in vitro* of Maren *et al* (7).

The slight and transient effect of a small dose of this compound in the dog is shown in Fig. 1, the plasma concentration drops rapidly, alkalization of the urine is brief, and the drug output in the urine almost nil. A direct comparison with acetazolamide is available, since Maren *et al* ((6), Fig. 3, C) show the high renal recovery and relatively prolonged plasma

<sup>1</sup> However, it should be pointed out that it has rarely been possible to account quantitatively for the intact sulfonamide grouping among the excreted products of an administered sulfonamide compound.



concentrations following the same dose of acetazolamide in the same dog, made within a month of the experiment of Fig 1

One possible explanation for the results with benzothiazolesulfonamide would be the rapid and nearly complete metabolism of the compound by the dog. Two experiments were carried out in an attempt to verify this hypothesis by examination of the urine. Although these studies failed to account for the major portion of the sulfonamide, a metabolic product was isolated and characterized, accounting for 25 per cent of the administered

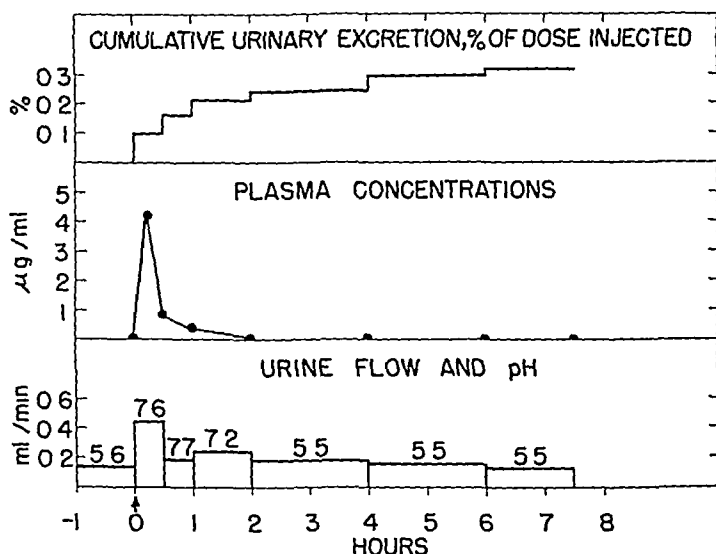


FIG 1 The transient renal effect and fate of 2-benzothiazolesulfonamide in the dog 5 mg per kilo intravenously at the arrow Dog M52, March, 1952 These data were obtained by Dr T H Maren of these laboratories, and are presented with his kind permission Analyses by the Maren method (7), which assays a compound in terms of its potency in inhibiting carbonic anhydrase The data are related to a standard curve to give actual concentrations of the compound (or its equivalent)

drug in the first case and 9 per cent in the second (26 per cent estimated by spectrophotometry to be present in the latter) This product was identified as the glucuronide of 2-mercaptobenzothiazole

## EXPERIMENTAL

### *Materials and Methods*

The dogs were healthy adult beagles, members of the colony maintained at these laboratories Urine was collected in bottles partially filled with mineral oil, placed under metabolism cages

Samples from the same batch of benzothiazolesulfonamide were used for both experiments The compound showed a sharp melting point, 176–177° (corrected), decomposed A Kjeldahl analysis gave 12.92 per cent N,

calculated, for  $C_7H_6N_2O_2S_2$ , 13.08 per cent. The possibility that this batch was contaminated with a significant amount of 2-mercaptobenzothiazole was excluded by the demonstration that the ultraviolet absorption spectrum of the batch employed (maxima at 274 and 237  $m\mu$  in phosphate buffer, pH 7.0) was superimposable on that of a highly purified sample, while a mixture of 95 per cent sulfonamide and 5 per cent mercapto compound gave a curve with a very evident new shoulder in the region of 320  $m\mu$ . (The pure mercapto compound in buffer, pH 7, shows an absorption maximum at 313  $m\mu$ .)

The ultraviolet absorption spectra were obtained with a Beckman model DU spectrophotometer, 1 cm quartz cells were used. Assays for metabolic products were carried out by accurate dilutions of unknown solutions and measurement of absorption spectra in the range, 220 to 370  $m\mu$ . Concentrations were estimated from the absorption maximum at 278  $m\mu$  shown by the pure metabolite ( $\epsilon_M = 12,700$ ). Actual weights were measured directly, except where noted.

### *Isolation of Metabolic Product*

*Male Dog M70, Weight, 17.4 Kilos*—A filtered solution of 2.50 gm of benzothiazolesulfonamide in about 20 ml of water containing 1 equivalent of NaOH was injected intravenously. The total 0 to 45 hour urine (415 ml) was diluted to 440 ml, filtered, and refrigerated. Portions were extracted with various solvents at neutral, acid, and basic pH levels. These extractions gave traces of 2-hydroxybenzothiazole (about 0.1 gm, m p 137–139°, Hofmann (8), m p 136°) and 2-mercaptobenzothiazole (after heating with acid), which were identified by melting point and mixed melting point with authentic samples, and by comparison of infrared and ultraviolet absorption spectra.

The final portion of this urine, 128 ml, was charged on a  $15 \times 107$  mm slurry-packed column of activated carbon (Darco G-60). The column was washed with 130 ml of water, then 150 ml of 0.1 N NaOH, and finally another 150 ml of water. The ultraviolet absorption of the effluents indicated that the metabolic product had been effectively taken up from the urine, and that little had been eluted during the washing. Elution with 95 per cent ethanol was followed in successive small fractions until the concentration of metabolite in the eluate had fallen by over 90 per cent from the peak value. The eluates were combined in three pools, and each was evaporated to a stiff brown gum, total weight, 440 mg.

Crystallization was achieved by dissolving the crude material with 2.5 parts of water and diluting with 2 volumes of acetone. Careful purification of one pool in this way resulted in recovery of 110 mg of white crystals from 203 mg of crude material after three precipitations. Weight losses

on repeated treatment were measured, and, after the product had been identified as the sodium salt of the glucuronide of 2-mercaptobenzothiazole (molecular weight, 365.3) containing 1 molecule of acetone of solvation (total molecular weight, 423.4), it was possible to calculate that the crude product was 70 per cent pure. On this basis, the 440 mg of 70 per cent pure material actually contained 308 mg of metabolic product (acetone free). Extrapolation to the total volume of pooled urine indicated that 1.06 gm would have been obtainable by this process, or the equivalent of 0.63 gm (25 per cent) of the administered sulfonamide.

*Male Dog M80, Weight, 14.5 Kilos*—The same amount of drug was injected in the same way. The urine was collected fractionwise (0 to 6, 6 to 24, 24 to 30, 30 to 45 hours) in this case, and each fraction was assayed spectrophotometrically. The 6 to 24 hour specimen, volume 520 ml, contained >95 per cent of the metabolite excreted. The estimated quantity was about 1.1 gm, equivalent to 0.64 gm of sulfonamide or about 26 per cent of the dose.

Since the original isolation technique appeared to be primarily an adsorption-elution process, the 520 ml of urine were filtered and stirred with successive portions of 3.0, 3.0, 4.0, and 5.0 gm of Darco G-60, to complete adsorption. The combined Darco adsorbates were washed twice with water, with an estimated loss of 15 per cent of the metabolite, and were then packed in a thick suspension in water into a 27 × 102 mm column. Elution with 500 ml of 95 per cent ethanol gave a poor recovery of the metabolite, along with another substance which had an absorption maximum at about 320 mμ, subsequently identified as 2-mercaptobenzothiazole. Since this maximum had not appeared in the spectrum of the untreated urine, the data suggested that partial hydrolysis of the glucuronide may have occurred on the Darco in this instance.

The column was washed with 40 ml of water, then 50 ml of 0.1 N NaOH, and a large volume of water. Elution was repeated with 1300 ml more of 95 per cent ethanol. Further amounts of glucuronide and of mercapto compound were obtained, but rough estimates from ultraviolet data indicated that little more than half of the glucuronide in the urine, at the most, was accounted for in the total eluate.

The eluate fractions were combined in two pools, according to the relative amounts of glucuronide and mercapto compound in each, and were evaporated. The free mercapto compound was first recovered from its pool by redissolving the crude material in a little water, mercaptobenzothiazole is insoluble, and was obtained practically pure by filtration and washing. Yield, 69 mg. The glucuronide was recovered as the acetone solvate, as described above. Yield, 260 mg. These materials together are equivalent to 219 mg of administered sulfonamide, or 8.8 per cent of the dose.

*Characterization of Metabolite*—Analytical results (m p 200–205° (corrected) decomposed) indicated an empirical formula

$C_{16}H_{18}NO_7S_2Na$	Calculated	C 45.4, H 4.3, N 3.3, S 15.1, Na 5.4
	Found	" 45.0, " 4.3, " 3.5, " 14.8, " 6.0 (from ash)

A 2 mg sample of the product was heated in 0.5 ml of 1 N HCl for 0.5 hour at 100°. Cooling gave white needles, m p 181–182° (corrected), not depressed by mixture with an authentic sample of 2-mercaptobenzothia-

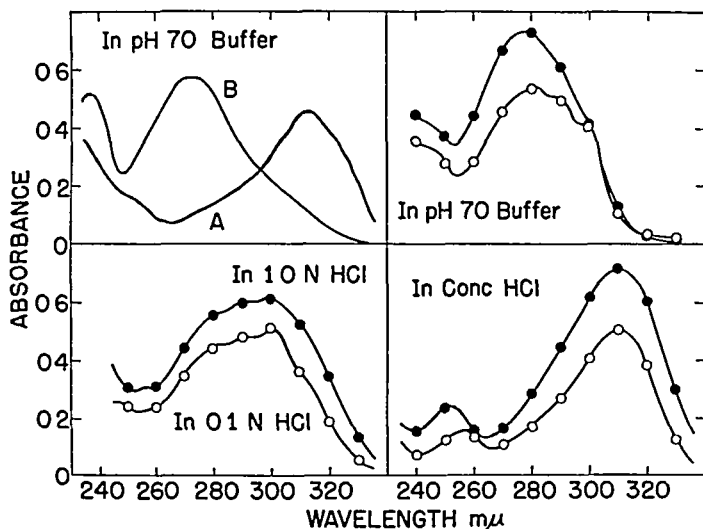


FIG 2 Ultraviolet absorption spectra (concentrations unknown) ●, the metabolic product, ○, 2-methylmercaptobenzothiazole, Curve A, 2-mercaptobenzothiazole, Curve B, 2-benzothiazolesulfonamide. The spectral shift of the metabolic product in dilute acid was measured in 1.0 N HCl because no significant shift was observed in 0.1 N HCl. The methylmercapto compound, on the other hand, showed the shift in the more dilute acid, as indicated.

zole, infrared and ultraviolet spectra were identical with those of an authentic sample.

The supernatant fluid from the hydrolysis gave a negative test with  $Ba^{++}$ , thus ruling out an "etheral sulfate". A positive test for glucuronic acid was obtained by the naphthoresorcinol method (9). A semiquantitative test for acetone was carried out by the salicylaldehyde procedure (10). An aliquot of the supernatant fluid calculated to contain about 0.13 mg of acetone gave approximately the same color as a control test with 0.1 mg of acetone. (2-Mercaptobenzothiazole and galacturonic acid gave negative results at the 1 mg level.)

The carboxyl group of glucuronic acid in the product was indicated to be unesterified by the presence of sodium, presumably as the salt of a fairly strong acid, since a water solution was approximately neutral. An electrometric titration confirmed the presence of a moderately strong acid.

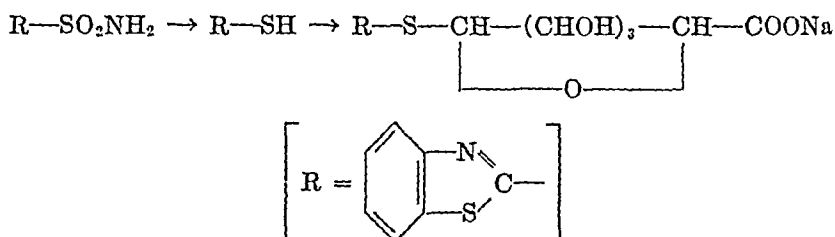
( $pK_a$  3.2 to 3.4), glucuronides have been found to have  $pK_a$  values in the range, 3.0 to 4.0 (11)

Evidence for the thio ether linkage in the product was furnished by the close correspondence of ultraviolet absorption spectra at different pH values between the metabolic product and an authentic sample of 2-methylmercaptobenzothiazole (Fig. 2). 2-Mercaptobenzothiazole, on the other hand, has a much higher principal maximum at pH 7 (313 versus 278  $m\mu$ ), and this does not shift below 308 even at a pH as high as 10.6. It thus seems most unlikely that the glucuronic acid residue is attached at any position other than the 2-mercapto group.

It was hoped that the typical attachment at the C-6 carbon of the glucuronic acid could be further demonstrated by a test with Benedict's reagent before and after acid hydrolysis, since "the ether type (linkage of glucuronic acid) is usually stable to alkali and non-reducing to alkaline reagents" (12). However, the intact metabolite reacted positively in the Benedict test. This result was therefore judged to be due to breaking of the thio ether linkage in the hot alkali.

#### DISCUSSION

2-Benzothiazolesulfonamide appears to be degraded by the dog as follows



The experimental results are believed to provide substantial proof for formulation of the metabolic product, as isolated, as the acetone solvate of the glucuronide shown.

Reduction of a sulfonamide group *in vivo* has not been reported in the literature to our knowledge, and appears to be a hitherto unrecognized metabolic pathway for sulfonamides. It should be emphasized that sulfonamides of the type studied here are often less stable chemically than ordinary aromatic sulfonamides and might be considered more susceptible to metabolic alteration as well.

The excretion of a mercapto compound conjugated with glucuronic acid is also apparently unreported, although alcohols and phenols are commonly handled thus in the body (13).

The fate of the large fraction of administered drug that was not ac

counted for remains unknown, degradation to a form which did not absorb in the region of 200 to 400  $m\mu$  would have been unrecognized in this work

The metabolite isolated is apparently produced and excreted at a moderate rate, being undetectable in the ultraviolet spectrum of the urine after 24 hours. Furthermore, there was no evidence for a significant quantity of any other metabolic product of the drug in the spectrum of the urine at any time up to 48 hours, so that any release from a depot form seems unlikely

Much of this work was carried out in the laboratory of Dr P H Bell, and I acknowledge with pleasure his many helpful suggestions and discussions. I also gratefully acknowledge the assistance of the Pharmacology Laboratory under Dr T H Maren of the Experimental Therapeutics Research Section, where all of the work on animals and the bioassays were carried out, of the Infrared Spectroscopy Group under Dr R C Gore for measurement and interpretation of infrared spectra, of the Microanalytical Laboratory under Dr J A Kuck for the microanalyses, and of Miss V W Bliss in the chemical work

#### SUMMARY

When 2-benzothiazolesulfonamide is administered intravenously to the normal dog, it is metabolized, at least in part, to a compound identified as the glucuronide of 2-mercaptobenzothiazole. The actual fraction of the dose converted to this form is uncertain, but no evidence was found in the urine for intact drug or other metabolic products

Reduction of a sulfonamide and conjugation of a mercapto compound with glucuronic acid appear to be previously unrecognized metabolic processes

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# CHROMATOGRAPHIC FRACTIONATION OF BEEF ADRENAL EXTRACT

## I ISOLATION OF ALDOSTERONE ✓

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In previous publications we have described the isolation of a sodium-retaining steroid from beef adrenal glands (1) and from the amorphous fraction (2) of a beef adrenal extract. The sodium-retaining steroid was shown to be identical with the aldosterone isolated by Simpson and associates (3). It is the purpose of this communication to describe an improved procedure for the isolation of aldosterone. Observations on the effect of the combination of silica gel and certain organic solvents on cortisone and hydrocortisone are included, as well as evidence that a monoacetate previously described is aldosterone-11,18-lactol 18-acetate.

Aldosterone was isolated from two different beef adrenal extracts, one being prepared by the use of acetone (4) and the other through the use of alcohol (5, 6). The acetone<sup>1</sup> extract contained a large amount of diacetone alcohol and of dark amorphous material which greatly increased the difficulties of fractionation. Chromatography of the oily residue (67 gm.) from the acetone extract of 1200 pounds of glands on a column of silica gel in formamide-benzene, then on paper in formamide-benzene, and finally on paper in propylene glycol-toluene gave 23.7 mg. of aldosterone.

The alcoholic extract from 2060 pounds of adrenal glands was almost colorless, its dry weight being only 7.8 gm. Fractionation and crystallization of the individual components were much easier in the case of the alco-

\* The Mayo Foundation, Rochester, Minnesota, is a part of the Graduate School of the University of Minnesota.

<sup>1</sup> When the acetone extract of 1200 pounds of glands was concentrated as far as possible at room temperature and about 15 mm. pressure, the residue weighed 218 gm. After further concentration at 0.03 mm. pressure and 30°, in conjunction with a receiver cooled with dry ice and methanol, the weight was reduced to 67 gm. The distillate obtained during the latter concentration was identified as diacetone alcohol by means of its boiling point and by the melting points and mixture melting points of its semicarbazone, *p*-nitrophenylhydrazone, and 2,4-dinitrophenylhydrazone with authentic specimens of these derivatives of diacetone alcohol. Diacetone alcohol also has been encountered in the fractionation of an acetone extract of adrenal glands by Simpson and associates (3).



holic extract than of the extract with acetone. The fractionation of the alcoholic extract is presented in detail.

During experiments designed to improve the packing of silica gel columns and the resolution of mixtures of steroids, it was found that the recovery of cortisone and hydrocortisone was poor from columns of silica gel with formamide and 1:1 chloroform-benzene or columns of silica gel with formamide and benzene. In model experiments which simulated the conditions of column chromatography, cortisone and hydrocortisone were converted in part to adrenosterone and  $11\beta$ -hydroxy- $\Delta^4$ -androstene-3, 17-dione, respectively. In similar experiments in which powdered paper was substituted for silica gel, no products formed by loss of the side chain from cortisone and hydrocortisone were detected.

Since the primary purpose of this investigation was the isolation of aldosterone, conditions were sought which would separate it from the other components of the extract. In formamide with 1:1 chloroform-benzene, aldosterone migrates 0.80 as fast as cortisone on sheets of paper. Chromatography of the residue derived from the alcoholic adrenal extract on a paper column in the system formamide with 1:1 chloroform-benzene for 6 days separated aldosterone from all of the major steroids of the extract except cortisone. The steroids more mobile than cortisone were resolved to such an extent that those present in moderate quantity (Kendall's Compounds<sup>2</sup> A, B, and H and Reichstem's Substances P and R) could be isolated without repeated chromatography. The fractions which were less mobile than cortisone moved so slowly that it appeared that an unusually long time would be required to remove all of the steroids from the column. In an attempt to accelerate elution of the steroids less mobile than aldosterone, the mobile phase was changed from 1:1 chloroform-benzene to chloroform.<sup>3</sup> The change in the composition of the mobile phase was made gradually through use of the apparatus described in the

<sup>2</sup> The following trivial names are used: Compound A (11-dehydrocorticosterone), Compound B (corticosterone), Compound H ( $3\beta$ , 21-dihydroxyallopregnane-11, 20-dione), Substance P ( $3\beta$ ,  $17\alpha$ , 21-trihydroxyallopregnan-20-one), Substance R ( $3\beta$ ,  $11\beta$ , 21-trihydroxyallopregnan-20-one), Compound E (cortisone), Substance K (allopregnane- $3\beta$ ,  $17\alpha$ , 20 $\beta$ , 21-tetrol), Compound F (cortisol), Substance D ( $3\beta$ ,  $17\alpha$ , 21-trihydroxyallopregnane-11, 20-dione), Compound C ( $3\alpha$ ,  $11\beta$ ,  $17\alpha$ , 21-tetrahydroxyallopregnan-20-one).

<sup>3</sup> Unfortunately this procedure caused poor resolution of the steroids which were less mobile than cortisone. It was determined subsequently by the use of a dye (Sudan III) that in such a procedure the denser chloroform layer tends to run down one side of the column and may transform a narrow horizontal band at the top of the column into an oblique band which extends over half the length of the total column. On the other hand, it was found that, if the mobile phase was changed from chloroform to benzene (denser to less dense), a band at the interface moved down the column very evenly.

experimental section Chromatography was continued until 1300 fractions had been eluted and until the amount of steroid per fraction, as measured

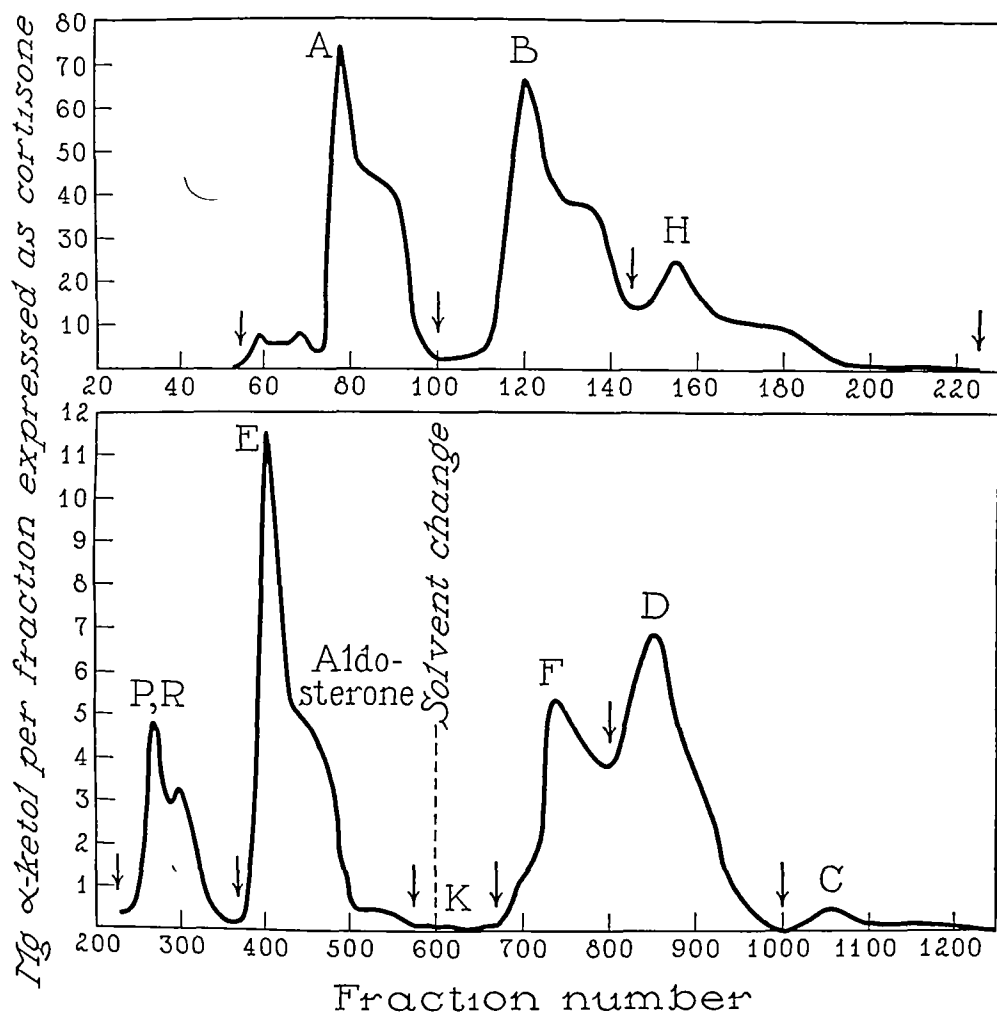


FIG 1 Pattern of chromatographic fractionation of an adrenal extract on a column of powdered paper The amount of  $\alpha$ -ketolic steroid per fraction was determined by use of blue tetrazolium and expressed as mg of cortisone Each fraction in the region of Fractions 50 to 85 was analyzed, every fifth subsequent fraction was analyzed Note the difference in scale of the two panels Solvent system, formamide with 1 l chloroform-benzene At Fraction 601, the mobile phase was changed to chloroform Kendall's Compounds A, B, C, E, F, and H and Reichstein's Substances D, K, P, and R were present in the regions indicated by these letters and set off by the arrows on the graphs

by the reaction with blue tetrazolium, was less than 0.05 mg The final fractions eluted contained a small amount of Kendall's Compound C Whether this extract contained substances less mobile than Compound C

is not known, since, if they had been present, they would not have been eluted from the column

Progress of the fractionation was followed by estimating the content of  $\alpha$ -ketol in the fractions and plotting this value against the fraction number as shown in Fig 1. The contour of the graph was used as a guide in combining fractions from the column. An aliquot of Fraction 405 was chromatographed on a sheet of paper and shown to contain cortisone. Assuming that this fraction contained the maximal concentration of cortisone, it was estimated that the peak concentration of aldosterone should be in Fraction 505 if aldosterone migrates 0.80 as fast as cortisone. The weights of aldosterone isolated from the various fractions indicate that the peak concentration of aldosterone occurred at some fraction earlier than 505. Individual fractions were combined as follows: Fractions 371 to 404, 405 to 455, 456 to 505, 506 to 575, and 576 to 670. After as much cortisone as

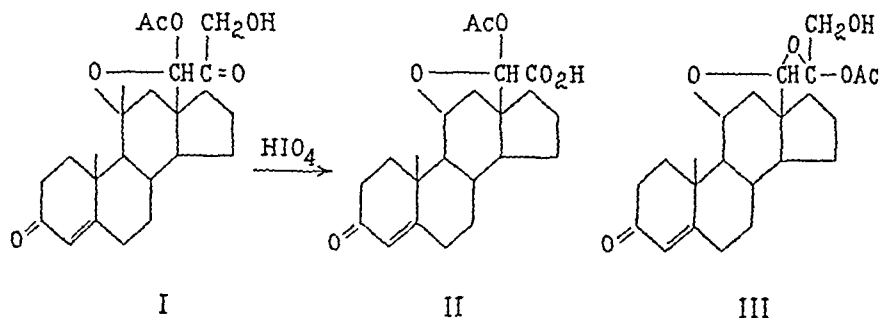


FIG 2

possible was removed from the various lots by crystallization from chloroform, the large fractions were chromatographed on sheets of paper in formamide-benzene. The zones corresponding in mobility to aldosterone were cut out, the steroid was removed by washing with alcohol, and crystals of aldosterone were obtained from acetone-water. After the mother liquors were chromatographed, the total aldosterone obtained from the 2060 pounds of glands was 44.7 mg. The total amount of cortisone obtained from the extract weighed 776 mg.

The previously described monoacetate (1), m.p. 217–219°, which was obtained by enzymic hydrolysis of aldosterone diacetate, has been designated (2) aldosterone-11,18-lactol 18-acetate (structure I, Fig 2). This structure was based on the following considerations. The diacetate from which the acetate (I) was derived has been formulated as aldosterone 11,18-lactol 18,21-diacetate by Simpson and coworkers (3). Since the acetate (I) differs from the 21-monoacetate in chromatographic mobility, melting point, and sodium-retaining activity, it was concluded that structure I must be the 18-acetate unless the enzyme caused some rearrange-

ment The following observations substantiate structure I The infrared spectrum<sup>4</sup> of the substance in chloroform shows bands at 3500, 1664, and 1612  $\text{cm}^{-1}$  which are ascribable to the C-21 hydroxyl group, the ketone at C-3, and the 4,5-double bond The band at 1746  $\text{cm}^{-1}$  is assigned to the acetyl group A band at 1708  $\text{cm}^{-1}$  is ascribable to a carbonyl group at C-20 Thus, any structure such as structure III, which has no carbonyl group corresponding to the absorption band at 1708  $\text{cm}^{-1}$ , is excluded as a possibility Oxidation of the monoacetate with periodate at pH 5.9 leads exclusively to an acidic substance formulated as structure II

#### EXPERIMENTAL

The solvents used were of analytical grade, and were distilled before use The formamide (Matheson, Coleman, and Bell, 99 per cent) was distilled at about 15 mm pressure in an apparatus which was designed so that the vacuum was not released between the collection of a small fore fraction, which always contained ammonia, and the main ammonia-free fraction When stored in the dark, the main fraction remained ammonia-free indefinitely The powdered paper was Whatman, standard grade, and the silica gel was Davison No 922, through 200 mesh Infrared spectra were obtained with a Perkin-Elmer model No 12C instrument A spiral mercury resonant lamp with a Corex No 9863 filter was used to provide illumination at 254  $\text{m}\mu$  for observing paper chromatograms and detecting  $\alpha,\beta$ -unsaturated ketones

*Action of Silica Gel on Cortisone and Hydrocortisone*—The steroids were treated under conditions to simulate those used during column chromatography The supporting medium (4.0 gm of silica gel or powdered paper) was mixed with 25 ml of the mobile phase (chloroform-benzene or benzene) in a glass-stoppered 50 ml Erlenmeyer flask, and 2.0 ml of formamide were added while the suspension was being stirred mechanically The steroid (about 5 mg) was added and the flask was stoppered, shaken by hand for several minutes, and placed in the dark for 5 days The suspension was diluted with 200 ml of chloroform and 20 ml of water, shaken vigorously, filtered, and washed with 25 ml of chloroform The chloroform solution was washed twice with 20 ml portions of water and taken to dryness *in vacuo* The amount of steroid recovered was estimated from the absorption at 238  $\text{m}\mu$  by using  $\epsilon = 16,500$  as a measure of the 3-keto- $\Delta^4$  chromophore, and the amount of  $\alpha$ -ketol was estimated through the use of blue tetrazolum When cortisone was used, the recovery indicated by the absorption at 238  $\text{m}\mu$  was 94 and 89 per cent from powdered paper and silica gel, respectively, whereas the recovery of  $\alpha$ -ketol was 89 and 38 per

<sup>4</sup> We are indebted to Dr R. Norman Jones, National Research Council of Canada, for the infrared spectrum and its interpretation

cent Aliquots of the paper-treated and silica-treated samples of cortisone were chromatographed on paper in formamide 1:1 cyclohexane-benzene for 7 hours. The silica-treated sample contained a substance which moved at the same rate as adrenosterone. After the mixture was chromatographed in formamide 1:1 cyclohexane-benzene for 10 hours, the zone which moved at the same rate as adrenosterone was eluted, crystallized from acetone-ether, and identified by m.p. 219–220°, by mixture melting point (no depression) with adrenosterone, and by the identity of its infrared spectrum in chloroform with that of adrenosterone. Another substance which moved about 0.57 as fast as adrenosterone was present in smaller amount. In the paper-treated sample, no product moving faster than cortisone was observed.

In a similar manner a sample of hydrocortisone was treated with formamide and benzene (without chloroform) on paper and on silica gel for 5 days. The recovery indicated by absorption at 238 m $\mu$  was 97 and 87 per cent from powdered paper and silica gel, respectively, the recovery of  $\alpha$ -ketol was 81 and 9.6 per cent. Paper chromatography (formamide with 1:1 cyclohexane-benzene, 16 hours) of the product from the silica gel treatment of hydrocortisone gave an ultraviolet-absorbing zone at 6.5 to 9.6 cm from the origin. The substance extracted from this zone with alcohol melted at 196–198° after crystallization from acetone-ether. It did not depress the melting point of 11 $\beta$ -hydroxy- $\Delta^4$ -androstene-3,17-dione, and its infrared spectrum in chloroform was identical with that of 11 $\beta$ -hydroxy- $\Delta^4$ -androstene-3,17-dione.

*Adrenal Extract*—An extract prepared from 2060 pounds of beef adrenal glands by the procedure of Kendall (5, 6) had been stored at 4° in aqueous solution containing 0.1 per cent sodium chloride in dark bottles for 5 years. There was no mold present and the solution was practically colorless. Bioassay of an aliquot in adrenalectomized rats<sup>5</sup> indicated the presence of about 35 mg. of aldosterone. The solution was concentrated *in vacuo* at room temperature to 6.4 liters and extracted five times with 0.1 volume of chloroform and twice with 0.1 volume of ethyl acetate. The organic solutions were washed twice with 0.04 volume of water, combined, and evaporated to dryness *in vacuo*, total weight = 7.87 gm. The residue gave crystals at –18° from 14 ml. of chloroform. Recrystallization of the product from acetone gave 176 mg. of material, m.p. 212–218°, which was identified as cortisone by means of an infrared spectrum in Nujol and of a mixture melting point. The filtrates were taken to dryness to yield 7.70 gm. of residue, which was dissolved in 6.0 ml. of 1:1 chloroform-benzene and chromatographed.

<sup>5</sup> We are indebted to Dr. A. Albert for this assay. Determinations of sodium and potassium were made by Louise Reichrath.

*Packing Column with Paper*—Powdered paper (1285 gm) was added slowly to 11 liters of a 1:1 mixture of chloroform and benzene saturated with formamide, which was being stirred vigorously. To the suspension were added 642 ml of ammonia-free formamide over a period of several minutes with vigorous stirring. The suspension was stirred until it appeared to be uniform. The stopcock of a  $6.2 \times 100$  cm column was lubricated with formamide, the column was half filled with mobile phase (1:1 chloroform-benzene saturated with formamide), and a small wad of cotton was placed at the bottom of the column. An aliquot of the suspension of paper, sufficient to give a layer about 0.3 cm high after packing, was added, dispersed evenly, and packed tightly with a Howard and Martin type packer (7), which was 1 mm smaller in diameter than the column. Particular care was taken to have the column vertical and to pack the layers evenly and tightly. The column was packed to a height of 88.5 cm and 1008 gm of the powdered paper were used. The mobile phase, which had passed through the column, was slightly yellow and contained 76 mg of solids per liter after being washed with water and concentrated to dryness, it was discarded. The column was tested by applying 50 mg of Sudan III in 5.0 ml of mobile phase while the solvent flowed from the column at the rate of 1.55 ml per minute = 0.052 ml per sq cm per minute. Fractions (21.2 ml each) were collected at intervals of 13.7 minutes, and the band was eluted in Fractions 53 to 67, which represent 23 per cent of the volume which had passed through the column.

*Fractionation of Adrenal Extract*—While the mobile phase was running through the column at 1.64 ml per minute and the liquid was just level with the top of the paper column, the 7.70 gm of extract in 6.0 ml of mobile phase (1:1 chloroform-benzene saturated with formamide) were added. After the surface of the solution had descended to just below the level of the paper, several small portions of mobile phase were added to the top of the column to wash all of the extract into the paper. Mobile phase was added to a level of about 6 cm above the column of the paper, a wad of cotton was added to prevent the entering liquid from disturbing the surface, and a source of mobile phase was provided in a stoppered separatory funnel. Fractions which measured 22.6 ml each were collected at intervals of 13.75 minutes.

Progress of the chromatography was followed by determining the amount of  $\alpha$ -ketol in appropriate fractions. Aliquots (0.10 ml) were removed<sup>6</sup> and diluted with 0.7 ml of 95 per cent ethanol, 0.20 ml of 0.2 per cent ethanolic blue tetrazolium, and 0.50 ml of 0.10 N ethanolic choline. The solution was mixed after each addition. A blank and a standard which contained

<sup>6</sup> The aliquots from the more concentrated fractions had to be diluted with mobile phase before they were mixed with the other reactants.

10  $\gamma$  of cortisone were run simultaneously After 15 minutes at room temperature, the optical density was read at 530  $m\mu$  on a Coleman junior spectrophotometer The graph in Fig 1 shows the amount of  $\alpha$ -ketol, expressed as mg of cortisone per fraction, plotted against the fraction number

After 601 fractions had been collected, the mobile phase was removed from the top of the column A change in the composition of the mobile phase was provided through the use of two 500 ml separatory funnels To one funnel (A) were added 400 ml of 1:1 chloroform-benzene, which was saturated with formamide The stem of the other funnel (B), which had its stopcock closed, was introduced into Funnel A through a tight fitting rubber stopper in the neck of Funnel A Funnel B was completely filled with chloroform saturated with formamide, and the stopper was introduced and sealed with a few drops of formamide The joined funnels were then placed at the top of the column through a loose fitting stopper, and the stopcocks were opened After 5 hours, Funnel B was refilled with chloroform saturated with formamide After 9½ hours, the mobile phase entering the column contained about 94 per cent of chloroform, the mobile phase was removed from the top of the column and replaced with formamide-saturated chloroform For Fraction 695 and thereafter, 0.10 ml of chloroform saturated with formamide was used in the blank and in the cortisone standard for  $\alpha$ -ketol determinations

*Combining of Fractions*—Individual fractions were combined and treated soon after they had been eluted Fractions which contained cortisone or aldosterone or both were combined<sup>8</sup> as follows 371 to 404, 405 to 455, 456 to 505, 506 to 575, 576 to 670

The combined fractions were concentrated<sup>9</sup> *in vacuo* to oily residues Each residue was dissolved in 500 ml of chloroform, and the solution was washed with 50 ml of water The aqueous wash was extracted twice with 50 ml portions of chloroform and the organic phases were combined The chloroform solution was washed twice more with 50 ml portions of water and concentrated to an oil *in vacuo* and several small added portions of methanol were removed The combined fractions were stored at 4° in methanol

*Aldosterone Zone*—Fractions 371 to 404 yielded 125 mg of cortisone from cold chloroform, Fractions 405 to 455 and 456 to 505, with similar treat

<sup>7</sup> The use of this apparatus was suggested by Dr W J Haines and Dr R E Knauff

<sup>8</sup> The remaining fractions were combined as follows 54 to 101, 102 to 145, 146 to 220, 226 to 370, 671 to 800, 801 to 1000, 1001 to 1130, 1131 to 1300 Separation of crystalline compounds from these fractions will be described in a subsequent paper

<sup>9</sup> For the combined Fractions 801 to 1000 and higher, the preliminary concentration to dryness before washing with water was omitted

ment, gave 321 and 114 mg of cortisone. Crystallization of Fractions 576 to 670 gave 10.3 mg of product, m.p. 160–164°, which subsequently was shown to be Reichstein's Substance K. Fractions 506 to 575 and the filtrates from the fractions which yielded cortisone and Substance K were then chromatographed<sup>10</sup> separately on 40 × 55 cm sheets of Whatman No. 1 washed paper (1.4 to 1.7 mg of steroid per cm width of paper) in a Chromatocab in formamide-benzene for 100 hours. A 40  $\gamma$  sample of cortisone was included at the edge of each large sheet of paper as a standard. The chromatograms were dried in an air current, observed very briefly under 254 m $\mu$  illumination, and marked. The area containing substances which moved about 0.65 times the rate of the cortisone standard, which reduced tetrazolium (as shown by a test strip) and which appeared dark under 254 m $\mu$  illumination, was cut out and eluted with ethanol. The solvent was removed and the residue in chloroform was washed with water. The chloroform was removed *in vacuo* and crystals were obtained from a small volume of aqueous acetone. The following weights of crystals, which melted over a range of a few degrees at about 115°, solidified and remelted at about 157°, were obtained: Fractions 405 to 455, 0.7 mg; 456 to 505, 24.6 mg; 506 to 575, 10.7 mg; 576 to 670, 0.7 mg; no aldosterone was found in Fractions 371 to 404. After the aldosterone mother liquors from Fractions 405 to 670 were chromatographed in propylene glycol-toluene on paper for 112 hours, an additional 20.6 mg of crude aldosterone were obtained. Rechromatography of the cortisone zone of the formamide-benzene chromatograms yielded 40 mg of cortisone but no aldosterone.

Recrystallization of the various crops of crude aldosterone from acetone-water gave 44.7 mg of purified material (36.0 mg, m.p. 115–120° and 155–162°, 8.7 mg, m.p. 120–125° and 163–167°). The infrared spectrum of the compound in chloroform was identical with that of aldosterone.

The total cortisone isolated in Fractions 371 to 575, inclusive, amounted to 600 mg, the 176 mg which were crystallized from the whole extract before chromatography make the total yield 776 mg. It was identified by means of its melting point, chromatographic mobility, and infrared spectrum.

*Periodate Oxidation of Aldosterone-11,18-lactol 18-Acetate*—To 50  $\gamma$  of aldosterone-11,18-lactol 18-acetate in 0.5 ml of methanol, was added 0.5 ml of periodate solution. (The solution was prepared by dissolving 4.00 gm of H<sub>5</sub>IO<sub>6</sub> in 70 ml of water, adding 17.5 ml of 1.0 N sodium hydroxide solution, and diluting to 100 ml with water. A small amount of yellow precipitate formed, the pH of the solution was 5.9.) After 18 hours the solution was concentrated to about 0.3 ml in a stream of carbon dioxide,

<sup>10</sup> The technique of wetting the papers and running the chromatograms is outlined (2).



5 ml of ethyl acetate were added and the solution was washed three times with 0.3 ml of water. The organic phase was cooled to 4° and quickly washed three times with 0.3 ml portions of ice-cold 1.0 N aqueous sodium carbonate solution. The washings were collected in a flask which contained 3 ml of ethyl acetate and 10 ml of 1.0 N hydrochloric acid. The ethyl acetate phase, which had been washed with sodium carbonate, should contain the neutral steroids. It was washed twice with water, and taken to dryness and chromatographed on paper in formamide-chloroform until the solvent front reached the bottom of the paper. No dark spot was observed when the paper was examined under 254 m $\mu$  illumination. The ethyl acetate extract of the acidified sodium carbonate washings was washed three times with water, the organic phase was taken to dryness, and the residue was chromatographed on paper in formamide-chloroform until the solvent front reached the bottom of the paper. The paper was examined under 254 m $\mu$  illumination and a dark spot with  $R_F = 0.48$  was observed, on the same paper, 3,11-dioxo- $\Delta^4$ -etiamic acid had  $R_F = 0.36$ . Under the same conditions of oxidation and chromatography, aldosterone ( $R_F = 0.33$ ) gave a neutral fraction (the 18,20-lactone) with  $R_F = 0.85$  and no detectable acidic fraction.

#### SUMMARY

Cortisone and hydrocortisone were unstable in the presence of silica gel under conditions which initially were used for chromatography. Adrenosterone and 11 $\beta$ -hydroxy- $\Delta^4$ -androstene-3,17-dione, respectively, were the major products formed. Substitution of powdered paper for silica gel led to satisfactory recovery of cortisone and hydrocortisone.

An alcoholic extract of 2060 pounds of beef adrenal glands was fractionated on a column of powdered paper in formamide with a 1:1 mixture of benzene and chloroform. This procedure served to separate cortisone and aldosterone from the other major constituents of the extract. Crystallization of the mixture from chloroform removed most of the cortisone. Chromatography of the mother liquor on sheets of paper in formamide with benzene served to separate the remainder of the cortisone from the aldosterone. The extract from 2060 pounds of glands yielded 776 mg of cortisone and 44.7 mg of aldosterone.

A previously described aldosterone acetate, m.p. 217–219°, which is different from the 21-acetate, m.p. 193–195°, is formulated as aldosterone 11,18-lactol 18-acetate. This structure is supported by the infrared spectrum and by the formation of an acidic substance by oxidation with periodic acid.

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# THE EFFECT OF DIISOPROPYL FLUOROPHOSPHATE ON THE PROTEINASE AND ESTERASE ACTIVITIES OF THROMBIN AND ON PROTHROMBIN AND ITS ACTIVATORS\*

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Diisopropyl fluorophosphate (DFP) has been shown to inhibit a number of esterases (1, 2) and certain proteolytic enzymes possessing esterolytic activity (3). Balls and Jansen (4) have reported extensively on the stoichiometric nature of the reaction of DFP with chymotrypsin and trypsin, and the chemical composition of the enzymic centers with which DFP combines (5, 6) is under study in several laboratories.

Since the proteolytic nature of thrombin has been demonstrated in experiments which show the release of peptide material from fibrinogen (7, 8) and the fibrin clot (9), and its esterolytic role revealed by its activity in hydrolyzing esters such as *p*-toluenesulfonyl-L-arginine methyl ester (10), it seemed of interest to determine whether DFP would inhibit this protease-esterase. The results reported here show that DFP does inhibit the proteolytic and esterolytic activities of citrate-thrombin and biotrombin but has no effect on its precursor, prothrombin, or on the biological activators of prothrombin. Small amounts of DFP completely blocked the autocatalytic activation of prothrombin in 25 per cent sodium citrate solution.

## Materials and Methods

*Purified Prothrombin*—Purified prothrombin was prepared from bovine plasma by the methods of Seegers *et al* (11, 12). The preparations contained no detectable thrombin activity. The average prothrombin product contained about 150 mg of protein in a volume of from 15 to 25 ml. The average specific activity of these preparations was 23,000 units per mg of tyrosine (Folin and Ciocalteu) or 1400 units per mg, dry weight. Preparations of such specific activity are reported to be homogeneous in the ultracentrifuge (13).

*Citrate-Thrombin*—Thrombin was prepared from purified prothrombin

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samples by being activated in 25 per cent sodium citrate solutions at 37° until the reactions were 90 to 100 per cent complete. In some instances, the activations were accelerated by seeding the solutions with small quantities of thrombin and adding a few crystals of 6-methyl-2,4,4'-tri-amino diphenyl sulfone (14). After activation, each solution was diluted to 50 ml with distilled water, and the thrombin was precipitated by adding ammonium sulfate until the solution was 66 per cent saturated. The precipitate was dissolved and dialyzed against distilled water. These thrombin preparations are designated as "citrate-thrombin" (13).

*Biologically Activated Thrombin*—A 50 ml sample containing 75,000 units of prothrombin was activated by adding 2.5 ml of bovine lung thromboplastin suspension, 1.9 ml of 0.15 M  $\text{CaCl}_2$ , 0.5 ml of imidazole buffer, pH 7.2, and 0.1 ml of bovine serum previously adsorbed with barium carbonate. The activation process converted only 68 per cent of the prothrombin to thrombin and resulted in a product of low specific activity as compared to the citrate-thrombin materials prepared as described above. After centrifugation to remove the thromboplastin suspension, the thrombin was precipitated at 66 per cent ammonium sulfate saturation and the dissolved precipitate dialyzed. Thrombin prepared in this manner is designated as "biothrombin" (13).

*Dusopropyl Fluorophosphate*—DFP was kindly contributed by Dr. Bernard J. Jandorf of the Army Chemical Center, Maryland, and by Dr. Robert Sterner of Merck and Company, Inc., Rahway, New Jersey. Dilutions were made in anhydrous isopropanol.

*Prothrombin Determinations*—Prothrombin concentrations were measured by the modified two-stage method of Waie and Seegers (15). The prothrombin sample is first diluted to a low concentration and then activated by ionic calcium, tissue thromboplastin, and an accelerator source. The high dilution is important in reducing inhibitory effects. The prothrombin unit is that amount of precursor which yields 1 unit of thrombin.

*Thrombin Determinations*—Thrombin was determined according to the method of Waie and Seegers (15). The enzyme solution was diluted until it contained 1 "Iowa unit" per ml, i.e., the amount of thrombin which clots 1.0 ml of a standard fibrinogen solution in 15 seconds.

*Esterase Activity*—The ability of thrombin to split the ester linkage of the synthetic substrate, *p*-toluenesulfonyl-L-arginine methyl ester, thus yielding a free carboxyl group, was employed as a measure of the esterolytic activity of the thrombin. The procedure followed was that of Sherry and Troll (10), which defines the substrate unit as the number of micromoles of acid liberated in 10 minutes under standard conditions. The citrate thrombin preparations described above contained 6.7 clotting units per substrate unit.

*Miscellaneous Reagents*—A suspension of beef lung thromboplastin was prepared according to the procedures of Ware and Seegers (15). Bovine serum adsorbed with barium carbonate served as a prothrombin-free source of accelerator for the biological conversion of prothrombin. Imidazole buffer was prepared according to Mertz *et al* (16). Acetate, phosphate, Veronal, and glycine buffers of constant ionic strength were prepared by the methods outlined by Miller and Golder (17).

#### EXPERIMENTAL

*Effect of Diisopropyl Fluorophosphate on Purified Prothrombin*—To each of four tubes was added 1.0 ml of purified prothrombin solution in 0.15 M sodium chloride, containing 6000 prothrombin units and 0.64 mg of protein nitrogen, and also 0.5 ml of 0.1 N imidazole buffer, pH 7.2, and 0.4 ml of 0.15 M sodium chloride. 0.1 ml of anhydrous isopropanol was added to each of two of the tubes and  $2.7 \times 10^{-2}$  mmoles of DFP in 0.1 ml of isopropanol to the two remaining tubes. All solutions were maintained in a water bath at 37°. The prothrombin concentrations were measured at intervals throughout a 24 hour period. Since the two-stage prothrombin determinations involve the dilution of these solutions 2400 times at the moment they are activated to thrombin, the effectiveness of DFP on the thrombin formed during analysis is greatly reduced by the dilution. After 24 hours incubation with DFP and isopropanol, all of the original prothrombin activities were still measurable, indicating that DFP had no effect on the precursor.

*Inhibition of Thrombin by DFP*—After preliminary experiments which indicated that DFP inhibits thrombin, quantitative relationships between the amount of DFP and the degree of inhibition were studied. The inhibiting effect of DFP was measured not only upon the clotting activity of the thrombin but also upon its esterase activity, *p*-toluenesulfonyl-L-arginine methyl ester being used as substrate. Both citrate-thrombin and biothrombin preparations were employed as substrates for the inhibitor. These preparations were adjusted to contain 2640 clotting units and 395 synthetic substrate units per ml. The enzyme solvent was 0.15 M sodium chloride and was 0.01 N with respect to imidazole buffer, pH 7.2. The solution of citrate-thrombin contained 0.33 mg of protein nitrogen per ml.

The thrombin solutions were distributed in 1.0 ml volumes among a series of tubes and brought to 25°. 0.1 ml volumes of isopropanol containing varying amounts of DFP, or isopropanol alone, were added. Subsequently, the thrombin levels were followed by periodic analyses. Clotting and esterase measurements were made by withdrawing 0.1 ml samples and immediately diluting them in 0.15 M sodium chloride, thus slowing the inhibition reaction, and then assaying. In  $2.3 \times 10^{-3}$  M DFP,

the reaction was first order with respect to thrombin inhibition. This was not the case in the more dilute DFP solutions. Fig 1 shows the variation in the degree of inhibition of the clotting and esterase activities of the citrate-thrombin after 15 minutes incubation with varying amounts of DFP. Both enzymic activities were affected to the same extent as the DFP concentration was increased. In 15 minutes, 50 per cent of each enzymic

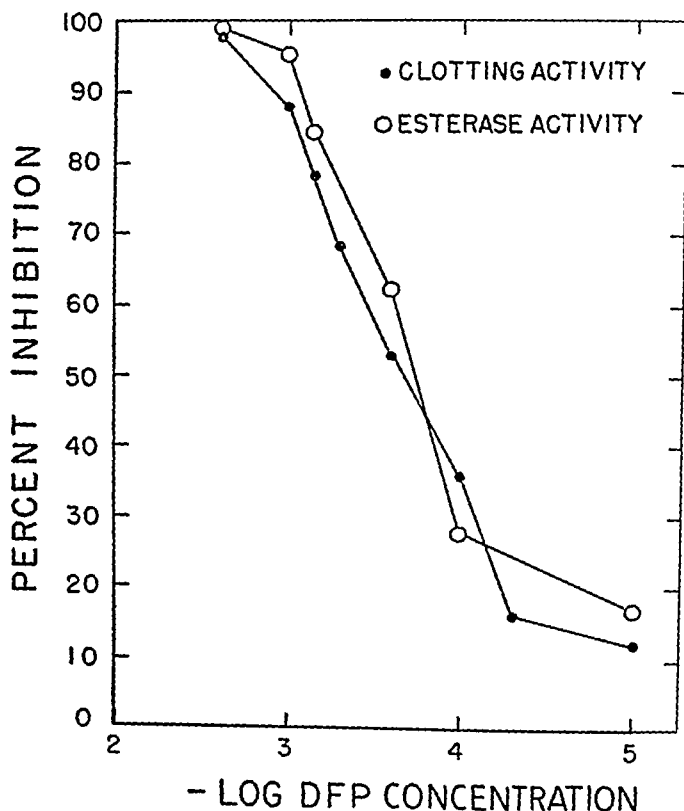


Fig 1 DFP inhibition of the clotting and esterase activities of citrate-thrombin. Initial thrombin concentration is 2400 units per ml in 0.15 M NaCl which is 0.01 M with respect to imidazole buffer, pH 7.2. Protein nitrogen, 0.30 mg per ml, initial esterase concentration, 360 *p*-toluenesulfonyl-L-arginine methyl ester units per ml, temperature, 25°, reaction time, 15 minutes.

activity was neutralized in  $2 \times 10^{-4}$  M DFP. Similar DFP inhibition curves were obtained for the clotting and esterase activities of biotrombin, although the latter was less pure than the citrate-thrombin. Half inhibition of the biotrombin was obtained in  $1 \times 10^{-4}$  M DFP after 15 minutes incubation.<sup>1</sup>

<sup>1</sup> A preliminary experiment was carried out to determine whether phosphorus<sup>32</sup> taken up by prothrombin, thrombin, or heat-denatured thrombin. A definite uptake was observed with citrate-thrombin but not with either of the other two proteins. To present significant quantitative data relating the moles of DFP coupled to

*Optimal pH for DFP-Thrombin Reaction*—Solutions of citrate-thrombin were prepared in a series of buffers acetate at pH 5.2, phosphate at pH 6.0 and 7.1, Veronal at pH 8.2 and 9.0, and glycine at pH 10.1. Thrombin is stable within the pH range covered (19). The ionic strength of each buffer was 0.10 and the thrombin concentrations were constant at 1750 clotting units per ml. To 1.0 ml samples was added 0.1 ml of either isopropanol or  $3 \times 10^{-3}$  M DFP in isopropanol. After 15 minutes at 25°, the

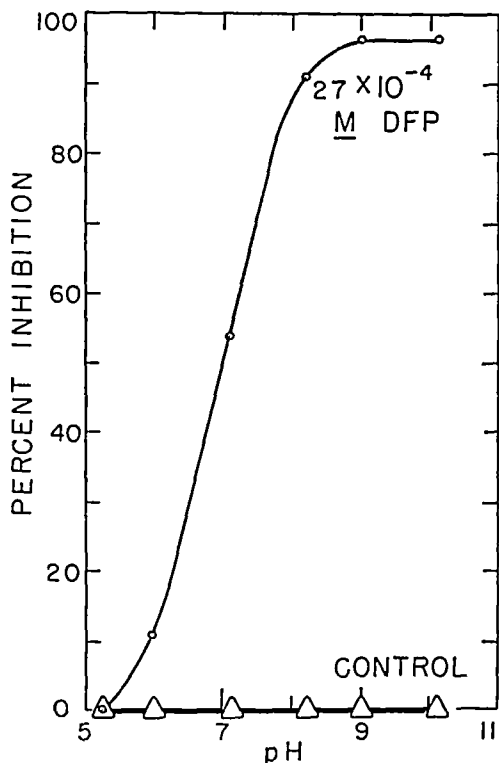


FIG 2 Influence of pH on the inhibition of citrate-thrombin by DFP. Initial thrombin concentration is 1600 units per ml. Initial DFP concentration is  $27 \times 10^{-4}$  M, ionic strength of all buffers, 0.10, temperature, 25°, reaction time, 15 minutes.

clotting activities of the mixtures were measured and the degrees of inhibition calculated. The results appear in Fig 2. It is obvious that the quantitative interaction between DFP and thrombin is dependent on pH. This effect is most marked between pH 6 and 8 under the conditions of this experiment. No inhibition was noted at pH 5.2, the inhibiting effect was maximal from about pH 8 to 10.

A mole of thrombin, the pure enzyme must be available. Since it is known that citrate-thrombin, although prepared from pure prothrombin, contains a mixture of active and inactive proteins (18), the stoichiometric study of this reaction must await a homogeneous thrombin preparation.



For quantitative studies which relate the amount of DFP to the amount of thrombin inhibited, pH 7.2 was selected because coagulation studies are made routinely at this pH. The sensitivity of the enzyme for the inhibitor could have been increased significantly if the coupling reaction had been carried out from about pH 8 to 10.

*Effect of DFP on Autocatalytic Activation of Purified Prothrombin in 25 Per Cent Sodium Citrate Solution*—The autocatalytic activation of prothrombin to thrombin in 25 per cent sodium citrate solution has been described as proceeding in two steps (20). The first is the formation of an inactive derivative plus trichloroacetic acid (TCA)-soluble materials. The second phase is the autocatalytic generation of thrombin. This reaction was studied in the presence of DFP to determine whether a dissociation of the prothrombin molecule actually took place because of the high salt concentration or whether traces of thrombin activity accounted for the observed results.

A sample of protein containing 564,000 prothrombin units in a volume of 40.0 ml. was cooled to 0°. It was then divided into two equal portions and 2.5 gm. of dry, powdered sodium citrate were added to each. 0.1 ml. of an anhydrous isopropanol solution containing  $2.7 \times 10^{-2}$  mmoles of DFP was added to one tube (A) and 0.1 ml. of isopropanol was added to the control tube (B). An additional 2.5 gm. of sodium citrate were then added to each solution. 2 ml. samples were periodically withdrawn from each tube and a fraction was analyzed for prothrombin and thrombin activity. The remainder was precipitated at 12 per cent TCA. Both the supernatant fluids and precipitates of the TCA fractionation were analyzed for nitrogen (Kjeldahl), tyrosine (Folin and Ciocalteu), and carbohydrate (anthrone reagent). At 6 hour intervals, 0.05 ml. of DFP ( $1.4 \times 10^{-2}$  mmoles) was added to tube A to replenish the DFP that may have hydrolyzed.

The results of these experiments are shown in Fig. 3. No TCA-soluble material was released in a system with DFP present, and the prothrombin activity was completely stable. The control experiments without the inhibitor demonstrated the formation of TCA-soluble material, the disappearance of prothrombin activity, and the formation of thrombin. The results observed in the controls could be attributed only to the presence of thrombin. This reaction appears to be autocatalytic and will be discussed later.

*Effect of DFP on Biological Activators of Prothrombin*—The complete inhibition of the activation of prothrombin in 25 per cent sodium citrate having been demonstrated, the following experiment was designed to determine whether the inhibitor affects a mixture of biological activators, including calcium ions, tissue thromboplastin, and dilute serum. Seven

tubes, containing a system of biological activators in the proportions given, were placed in a water bath at  $37^{\circ}$  (a) 0.15 M  $\text{CaCl}_2$ , 0.5 ml, (b) 0.1 N

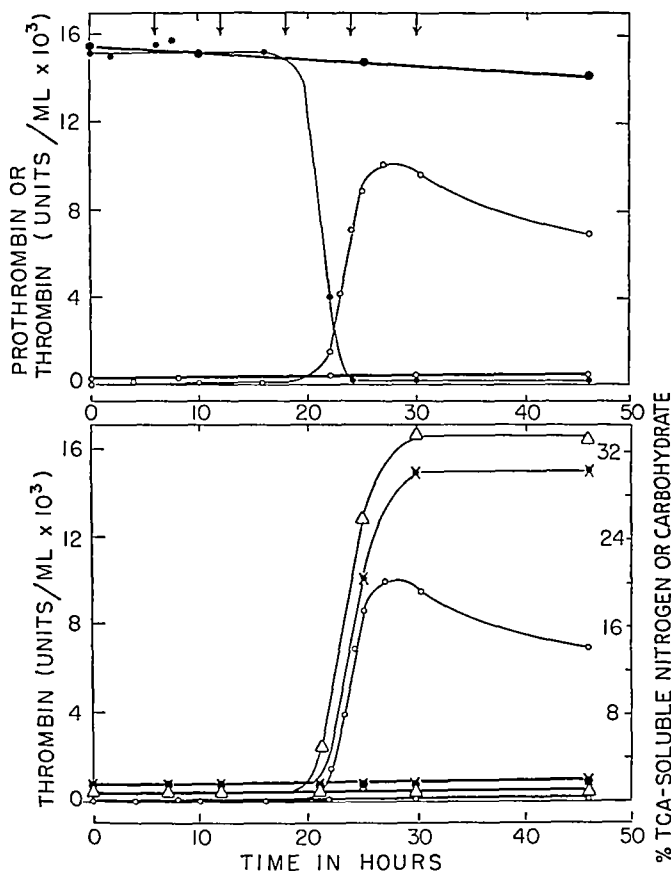


FIG 3 Incubation of purified prothrombin in 25 per cent sodium citrate solution with and without DFP Upper section changes in prothrombin and thrombin activities Lower section the same experiment, indicating changes in the nitrogen and carbohydrate components of the TCA-soluble fraction Temperature,  $37^{\circ}$ , initial volume, 20.0 ml, initial prothrombin concentration, 15,000 units per ml, initial nitrogen concentration, 1.3 mg per ml, initial DFP concentration,  $1.4 \times 10^{-3}$  M The heavy lines represent the DFP present, the light lines, the DFP absent Solid circle, prothrombin, open circle, thrombin, solid circle with cross, TCA-soluble nitrogen, open triangle, TCA-soluble carbohydrate, arrow,  $1.4 \times 10^{-2}$  mM DFP added in 0.05 ml volume (or 0.05 ml of isopropanol in the controls)

imidazole buffer, pH 7.2, 0.5 ml, (c) suspension of thromboplastin of lung, 0.5 ml, (d) accelerator serum diluted 1:50 in 0.15 M NaCl, 0.3 ml

To five of the tubes were added 0.2 ml volumes containing  $1.8 \times 10^{-2}$  mmoles of DFP To the remaining two tubes was added 0.2 ml of isopropanol All tubes were returned to the water bath except one which contained isopropanol To this activator mixture was added 1.0 ml of a

solution containing 5250 units of prothrombin in imidazole-buffered saline (1 volume of 0.1 N imidazole buffer, pH 7.2, to 3 volumes of 0.15 M NaCl). The activation of the prothrombin was followed by periodic determinations of thrombin. After 1, 4, 8, 16, and 40 hours at 37°, tubes containing the activator-DFP mixtures were removed from the water bath and 1.0 ml of the same prothrombin solution was added. Again the generation of thrombin

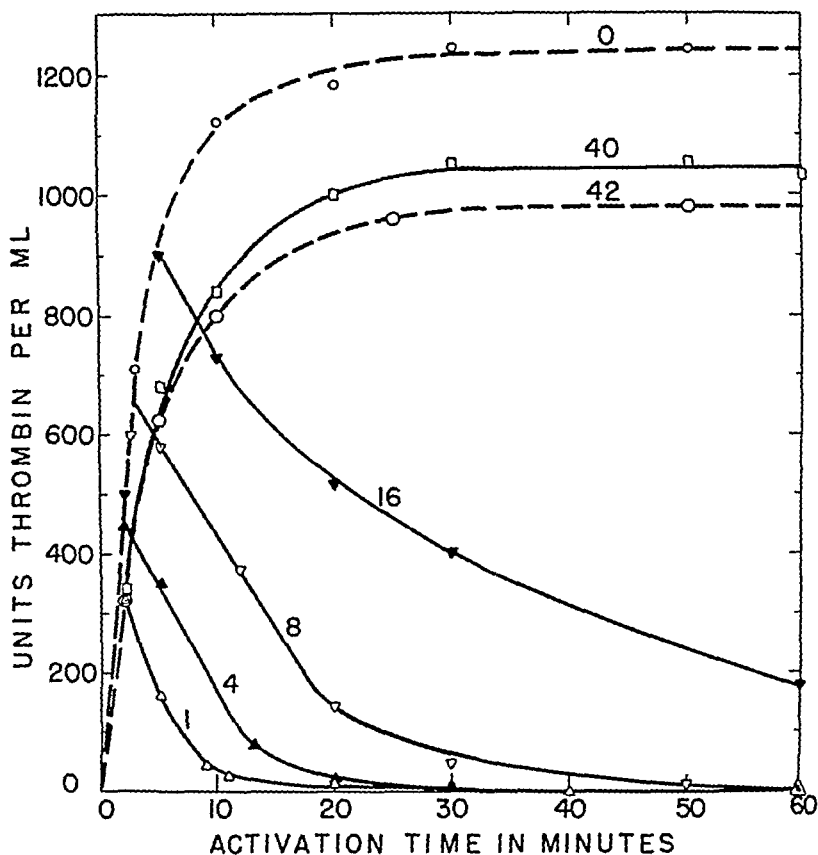


FIG. 4. Activation of purified prothrombin following incubation of the biological activators in  $9 \times 10^{-3}$  M DFP. The figures on the curve denote the number of hours that the biological activators remained in contact with DFP.

bin was followed by analysis. At 42 hours, the last activator-isopropanol mixture was removed and its prothrombin-activating capacity tested. The results of the experiment are depicted in Fig. 4.

The prolonged incubation of the activator mixtures was necessary to complete the hydrolysis of excess DFP which would inhibit the newly generated thrombin. The activator samples withdrawn before the hydrolysis of DFP was complete (*i.e.* 1, 4, 8, 16 hours) showed a partial, then increasing, ability to activate prothrombin. The secondary inhibition of thrombin observed during these periods was due to the inactivation of the

newly formed enzyme by residual traces of DFP. At 40 hours, the bio-activators exposed to DFP were as capable of activating prothrombin as those activators which had not been in contact with the inhibitor. Here, apparently, the hydrolysis of DFP was complete and there was no secondary inhibition reaction after thrombin generation.<sup>2</sup>

#### DISCUSSION

DFP was found to be without effect on prothrombin even when excessive amounts of the inhibitor were used (6000 prothrombin units in  $1.35 \times 10^{-2}$  M DFP). On the other hand, thrombin was rapidly inactivated by DFP. At pH 7.2, a thrombin solution containing 2400 units per ml had a half life of 15 minutes in  $2 \times 10^{-4}$  M DFP. The prothrombin-thrombin system resembles chymotrypsinogen-chymotrypsin in its reaction with DFP in that the precursor is non-reactive toward DFP while the enzyme is inhibited (3). This is of practical value in that prothrombin preparations can be saved from the deleterious effects of contaminating thrombin. Both the proteolytic and esterolytic activities of thrombin are inhibited to the same extent by equal amounts of DFP (Fig. 1) as are those of chymotrypsin, suggesting that a common center may be involved in both activities.

The reaction between thrombin and DFP is dependent on pH, as is the case with chymotrypsin and trypsin. The optimal pH for the coupling reaction, within the range of pH stability of the enzyme, appears to be between pH 8 and 10. In contrast, certain other esterases (1) are not dependent on pH for the DFP reaction.

According to the method used to activate prothrombin, several types of active thrombin can be formed. Among them are biotrombin and citrate-thrombin, which differ in their molecular weights, sugar content, etc. (13). DFP inhibits the clotting and esterase activities of the thrombins formed by either method of activation. Since citrate-thrombin preparations, although derived from physicochemically homogeneous prothrombin, are known to contain more than one active molecular species (13, 18), it is interesting to note that DFP does not discriminate among them. Apparently, the active moieties differ in the non-essential portions of their molecules.

When homogeneous thrombin preparations become available, the stoichiometric nature of the reaction may be studied by using isotopic DFP. Radioactive DFP- $P^{32}$ , combined with the newer techniques of peptide fractionation, promises to elucidate the chemical structure of the enzymic

<sup>2</sup> Just as Jansen *et al.* (21) have used the inhibition of chymotrypsin for following the hydrolysis of DFP preparations, the inhibition of thrombin has been used to indicate the strength of DFP solutions.

center which reacts with DFP. Serine phosphoric acid containing  $P^{32}$  has recently been isolated from digested diisopropylphosphoryl ( $P^{32}$ )-chymotrypsin (5). Other evidence suggests that the imidazole nucleus may be involved as an intermediate in the phosphorylation process (6).

The activation of purified prothrombin in 25 per cent sodium citrate solution has been studied extensively by Seegers and his coworkers. The activation was shown to take place at lower rates in lower citrate concentrations. The activation occurred with other salts and apparently the polyvalent anion was the most important factor in accelerating the activation process. Chloride appeared to be ineffective in activating prothrombin (22). Further investigations by the Seegers group into the reaction of prothrombin in 25 per cent sodium citrate indicated that considerable amounts of carbohydrate, nitrogen, and tyrosine were released into the TCA-soluble fraction in an early stage when the prothrombin was converted to an inactive derivative of lower molecular weight. Thrombin appeared in a second phase as a result of the autocatalytic activation of the prothrombin derivative (20). As shown in Fig 4, it is possible to stabilize prothrombin dissolved in 25 per cent sodium citrate by adding DFP. Neither the release of TCA-soluble material nor the formation of an inert prothrombin derivative was observed. All of the prothrombin activity remained after 36 hours in the citrate-DFP solution. With DFP, thrombin was irreversibly inactivated and, in the citrate activation mixtures, its level remained undetectable. Evidently the activation of prothrombin in the 25 per cent sodium citrate solutions is entirely autocatalytic. The dissociation phase may actually represent the first stages of hydrolysis in which small amounts of thrombin either become hyperactive or act upon bonds made more susceptible by the high ionic strength of the medium. The high ionic strength does not of itself dissociate the parent molecule.

#### SUMMARY

1 Thrombin was rapidly inactivated by very low concentrations of diisopropyl fluorophosphate (DFP). Prothrombin was unaffected even by excessive amounts of the inhibitor. The inhibition of the enzymic activities was observed, whether thrombin was produced by the bioactivation of prothrombin or by autocatalytic generation in high concentrations of sodium citrate.

2 Both the esterolytic and proteolytic activities of thrombin were affected equally by any given amount of inhibitor, suggesting that a common center may be involved in both activities.

3 The coupling reaction between thrombin and DFP is highly dependent upon pH with a maximal reaction between pH 8 and 10.

4 A system of biological activators of prothrombin (*i e* thromboplastin, serum accelerators, and ionic calcium) was unaffected by DFP

5 The activation of prothrombin in 25 per cent sodium citrate solution is completely blocked in the presence of DFP, establishing further the strict necessity for the presence of active thrombin in this activation process Prothrombin activity in 25 per cent citrate plus DFP solution was completely stable and no dissociation phase, that is formation of trichloroacetic acid-soluble products, was observed

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# SOME ASPECTS OF THE METABOLISM OF CHONDROITIN SULFATE-S<sup>35</sup> IN THE RAT

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The use of sulfate of exogenous origin in the synthesis of chondroitin sulfate was suggested (1) and then demonstrated by the isolation of chondroitin sulfate-S<sup>35</sup> from the cartilage of rats given sulfate-S<sup>35</sup> (2, 3). Confirmatory reports have supplemented the earlier observations (4, 5). Autoradiography has been particularly effective in showing that administered sulfate-S<sup>35</sup> is concentrated to a greater extent in regions which have a high acid mucopolysaccharide concentration (6-21) than in regions in which the concentration of such polysaccharides is low or in which they are absent. Results of experiments in which tissue cultures (22) and cartilage slices (23, 24) were used strongly suggest that the incorporation of sulfate into chondroitin sulfate is enzymatically controlled. It is likely that the reverse reaction, namely, the hydrolytic cleavage of the sulfate group from chondroitin sulfate, is similarly affected. However, the presence in mammalian tissues of an enzyme to catalyze such a reaction has not been demonstrated.

The following is a report of the fate of the sulfate group of chondroitin sulfate-S<sup>35</sup> when the latter is administered to rats by stomach tube or by intraperitoneal injection.

## EXPERIMENTAL

Adult male rats of the Sherman strain were used. Their average body weight was 310 gm, the range 290 to 330 gm. They were provided with Purina dog biscuits at all times except when urine was being collected.

In one set of experiments each of three rats received by stomach tube 3.3 mg of sodium sulfate-S<sup>35</sup> ( $2.90 \times 10^4$  c p m) in 1 ml of water<sup>1</sup>. The same amount of this material was injected intraperitoneally into each of three other rats. Each rat was then placed in a separate metabolism cage and allowed only water to drink. The urine excreted in the following 24 hours was analyzed for its content of inorganic sulfate sulfur, total sulfate sulfur, and total sulfur (25, 26). The S<sup>35</sup> in each of these fractions was also determined by assay of the barium sulfate samples after isolation on

<sup>1</sup> The S<sup>35</sup> used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.



filter paper disks (8) A week later the experiment was repeated with 20 mg of potassium chondroitin sulfate-S<sup>35</sup> ( $0.75 \text{ mg}$  of sulfur,  $2.70 \times 10^4 \text{ c p m}$ ) per rat and, after another week, with 25 mg of fresh S<sup>35</sup>-tagged cartilage ( $4.72 \times 10^3 \text{ c p m}$ ) homogenized in 1 ml of water The knee joints of 10 day-old rats that had received sodium sulfate-S<sup>35</sup> intraperitoneally 24 hours previously were the source of this cartilage

In a second set of experiments each of three different rats was given by stomach tube 100 mg of fresh S<sup>35</sup>-tagged cartilage ( $4.93 \times 10^4 \text{ c p m}$ ) homogenized in 1 ml of water and into each of three other rats the same amount of the material was injected intraperitoneally The urine excreted in the following 24 hours was collected and analyzed as in the first set of experiments A week later, the experiment was repeated with 10 mg of sodium chondroitin sulfate-S<sup>35</sup> ( $0.384 \text{ mg}$  of sulfur,  $3.66 \times 10^4 \text{ c p m}$ ) in 1 ml of water per rat

An additional series of experiments was done, again with rats of approximately the same body weight, with sodium chondroitin sulfate-S<sup>35</sup> of much greater specific activity than that of the preparations mentioned above Into each of three rats 1 mg of the chondroitin sulfate ( $1.82 \times 10^4 \text{ c p m}$ ) was injected intraperitoneally, into each of three rats in three other groups 2, 5, and 10 mg, respectively, were similarly injected Each of three additional rats received by intraperitoneal injection 10 mg of the chondroitin sulfate in 1 ml of a homogenate of 51 mg of fresh knee-joint cartilage, removed from 7 day-old rats The urines were collected and analyzed as above The unused portions of the urine samples from the rats that had received the same dose were pooled Portions of the pools of urine from the rats given 1 and 2 mg of the sodium chondroitin sulfate-S<sup>35</sup> were dialyzed against water in rocking dialyzers (27) at 0° for 24 hours and the dialyzable and non-dialyzable S<sup>35</sup> was determined The results obtained were checked by using urines immediately after collection from rats given 1 and 2 mg of the same sodium chondroitin sulfate-S<sup>35</sup>

In the case of the pooled urine samples from rats given 5 and 10 mg of the material, each of the pools in its entirety was dialyzed for a week against frequent changes of distilled water at 0° in bags of Visking casing The contents of the bags were then brought to dryness in small evaporating dishes kept in a vacuum desiccator over calcium chloride The residues were taken up in 1 ml of water and portions were used for analysis by paper chromatography according to Kerby (28) and by electrophoresis on paper and on starch (29, 30) To extend and verify the results, the urine excreted by a rat during a period of 17 hours following the intraperitoneal injection of 15 mg of sodium chondroitin sulfate-S<sup>35</sup> and the urine from a rat given similarly about  $100 \mu\text{c}$  of S<sup>35</sup> as sodium sulfate were analyzed as follows Each of these urine samples was diluted to 25 ml and of this 20

ml were then dialyzed for 1 week against frequent changes of distilled water at 0°, in bags of Visking casing. The non-dialyzable fraction of the urine was evaporated to dryness at room temperature over calcium chloride in a vacuum desiccator and the residue was taken up in 2 ml of water. Portions of the concentrate and of the undialyzed urine were analyzed by electrophoresis in a starch block. The concentrate was also examined by paper electrophoresis and paper chromatography.

As an adjunct to the above experiments the possible coprecipitation of barium chondroitin sulfate and barium sulfate was determined under the conditions used for the determination of the  $S^{35}$  in the inorganic sulfate sulfur fraction of the urines. The urine collected for 24 hours from two adult male rats was diluted to 100 ml with water. To 25 ml portions 1, 2, and 3 mg of sodium chondroitin sulfate- $S^{35}$  in 1, 2, and 3 ml of water, respectively, were added. A further dilution of each sample to 50 ml was made and, after mixing, two 5 ml portions were removed from each and further diluted to 100 ml with water, the addition of 5 ml of a 0.05 N solution of sodium sulfate, 1 ml of a 2.5 N solution of hydrochloric acid, and 5 ml of a 10 per cent solution of barium chloride followed. The barium sulfate was isolated 1 hour later by filtration onto paper disks for counting. After extensive acid hydrolysis of 5 ml portions of the urine samples, the total sulfate sulfur was isolated similarly. 24 hours later, the analyses were repeated, the urine samples having been kept at 20° during this period of time.

Skeletons of suckling rats, Sherman strain, that had received sodium sulfate- $S^{35}$  intraperitoneally at least 24 hours previously were the source of the chondroitin sulfate- $S^{35}$  used. Potassium chondroitin sulfate- $S^{35}$  was isolated according to a procedure reported by Einbinder and Schubert (31). On analysis it was found that it contained 2.86 per cent nitrogen, 3.75 per cent sulfate sulfur, 25.5 per cent hexuronic acid, and 20.4 per cent hexosamine. Sodium chondroitin sulfate- $S^{35}$  was prepared according to Bostrom (3). The preparation used in Experiments 9 and 10 (Table I) contained 3.96 per cent nitrogen, 3.84 per cent sulfate sulfur, 34.4 per cent hexuronic acid, and 25.9 per cent hexosamine, the preparation used in Experiments 11 through 15 contained 2.70 per cent nitrogen, 5.30 per cent sulfate sulfur, 32.4 per cent hexuronic acid, and 22.0 per cent hexosamine. The composition of the chondroitin sulfate preparations was ascertained as reported previously (5).

#### RESULTS AND DISCUSSION

The data on the excretion of  $S^{35}$  by the rats given sodium sulfate- $S^{35}$ , chondroitin sulfate- $S^{35}$ , and  $S^{35}$ -labeled cartilage are summarized in Table I. It can be seen that 52 to 86 per cent of the  $S^{35}$  administered as chondroitin

TABLE I

*Excretion of S<sup>35</sup> in Urine of Rats Given Chondroitin Sulfate S<sup>35</sup>*

The average of three values, each on a different rat, is listed in every instance  
Corrections for radioactive decay and self-absorption were made

Experiment No	Inorganic SO <sub>4</sub> -S		Total SO <sub>4</sub> -S		Total sulfur		Material and route of administration
	per cent of dose	c p m per mg S	per cent of dose	c p m per mg S	per cent of dose	c p m per mg S	
1*	62.5	2,110	73.9	2,160	68.8	1,178	3.3 mg Na <sub>2</sub> SO <sub>4</sub> -S <sup>35</sup> , stomach tube
2*	60.1	1,482	67.6	1,394	66.5	970	3.3 " " intraperitoneally
3†	45.3	1,074	52.0	1,055	51.3	741	20 mg K-chondroitin-SO <sub>4</sub> S <sup>35</sup> , stomach tube
4†	36.8	1,119	58.1	1,455	57.0	912	20 mg K-chondroitin-SO <sub>4</sub> S <sup>35</sup> , intraperitoneally
5‡	61.2	371	71.8	369	75.5	255	25 mg cartilage-S <sup>35</sup> , stomach tube
6‡	61.6	266	75.2	276	73.9	192	25 " " intraperitoneally
7§	61.6	3,108	70.7	3,041	72.4	2,215	100 mg cartilage-S <sup>35</sup> , stomach tube
8§	55.7	2,963	64.1	2,895	67.7	2,102	100 " " intraperitoneally
9	50.8	1,823	59.7	1,828	60.7	1,441	10 mg Na-chondroitin-SO <sub>4</sub> S <sup>35</sup> , stomach tube
10	54.2	2,182	85.9	2,914	86.0	2,417	10 mg Na-chondroitin-SO <sub>4</sub> S <sup>35</sup> , intraperitoneally
11¶	53.4	2,356	65.5	2,470	65.5	1,299	1 mg Na-chondroitin-SO <sub>4</sub> S <sup>35</sup> , intraperitoneally
12¶	52.0	3,977	67.6	4,165	69.6	2,857	2 mg Na-chondroitin-SO <sub>4</sub> S <sup>35</sup> , intraperitoneally
13¶	50.5	7,236	58.9	7,215	56.3	4,220	5 mg Na-chondroitin-SO <sub>4</sub> S <sup>35</sup> , intraperitoneally
14¶	44.2	10,402	65.4	12,530	62.6	8,615	10 mg Na-chondroitin-SO <sub>4</sub> S <sup>35</sup> , intraperitoneally
15¶	44.0	11,960	84.0	17,860	83.3	12,260	10 mg Na-chondroitin-SO <sub>4</sub> S <sup>35</sup> with 51 mg homogenized cartilage, intraperitoneally

\* Sodium sulfate was added to a solution of carrier-free sodium sulfate S<sup>35</sup> so that each ml contained 3.3 mg of the salt and  $2.90 \times 10^4$  c p m

† The 20 mg of potassium chondroitin sulfate-S<sup>35</sup> given to each animal were equivalent to  $2.70 \times 10^4$  c p m and 0.75 mg of sulfur

‡ 1 ml of a homogenate containing 25 mg of fresh knee-joint cartilage from 10 day-old rats was given to each of the adult rats. As a consequence of a previous administration of sodium sulfate-S<sup>35</sup> to the suckling rats, there were  $4.72 \times 10^5$  c p m of S<sup>35</sup> in 1 ml of the cartilage homogenate and 97.0 per cent of this activity did not pass through a cellophane membrane

§ Each of the rats was given 1 ml of a homogenate containing 100 mg of fresh knee-joint cartilage from 11 day-old rats that had previously received sodium sulfate-S<sup>35</sup>. There were  $4.93 \times 10^4$  c p m per ml of homogenate and of the activity 98.5 per cent did not pass through a cellophane membrane

TABLE I—*Concluded*

|| To each rat 10 mg of sodium chondroitin sulfate- $S^{35}$  were given, equivalent to 0.384 mg of sulfur and  $3.66 \times 10^4$  c p m

¶ Each mg of the sodium chondroitin sulfate- $S^{35}$  was equivalent to  $1.83 \times 10^4$  c p m. The material was isolated from the skeletons of 8 day-old rats that had received sodium sulfate- $S^{35}$  intraperitoneally 24 hours previously. For use in Experiment 15, the chondroitin sulfate- $S^{35}$  was dissolved in a homogenate of knee-joint cartilage removed from 7 day-old rats. Each ml of homogenate contained 10 mg of the labeled chondroitin sulfate and 51 mg of unlabeled cartilage.

sulfate- $S^{35}$  was excreted in 24 hours, the major portion, 37 to 62 per cent, was excreted as inorganic sulfate, and the remainder was found in the ester sulfate fraction. These values are not remarkably different from those found when sodium sulfate- $S^{35}$  or  $S^{35}$ -labeled cartilage was given. In the experiments in which sodium sulfate or fresh cartilage was administered by stomach tube or injected intraperitoneally, the specific activity of the sulfur, counts per minute per mg of sulfur, in the inorganic sulfate fraction of the urine was similar to that in the total sulfate fraction. This also appears to be the case when chondroitin sulfate was given by stomach tube, Experiments 3 and 9. On the other hand, when the chondroitin sulfate preparations were injected intraperitoneally, Experiments 4, 10, 14, and 15, the specific activity of the total sulfate sulfur in the urine was greater than that of the inorganic sulfate sulfur, except when the dose was 5 mg or less per rat, Experiments 11, 12, and 13. The results of Experiments 4, 10, 14, and 15 indicate that the specific activity of the ester sulfate sulfur was markedly higher than that of the inorganic sulfate sulfur. For example, one of the rats in Experiment 4 excreted 8.12 mg of inorganic sulfate sulfur and 10.15 mg of total sulfate sulfur in 24 hours and the radioactivity associated with these sulfate sulfur fractions was 9560 c p m and 15,560 c p m, respectively. The 2.04 mg of ester sulfate sulfur are therefore associated with a radioactivity of 6000 c p m, hence the specific activity of the ester sulfate sulfur is 6000 c p m per 2.04 mg or 2940 c p m as compared to 1177 c p m per mg of sulfur in the inorganic sulfate fraction. This and similar results obtained following intraperitoneal injection of more than 5 mg of chondroitin sulfate- $S^{35}$  are interpreted tentatively as follows. The rat is able to break the bond between the sulfate group and the rest of the chondroitin sulfate moiety, this capacity, however, is a limited one. If an excessive amount of chondroitin sulfate is injected, a significant amount of the sulfate is excreted without prior release from the carbohydrate unit to which it is attached.

The urines were dialyzed to ascertain the nature of the  $S^{35}$ -labeled material excreted in the ester sulfate fraction by rats given chondroitin sulfate intraperitoneally. Some of these data are presented in Table II. It was

found that following a dose of 1 mg practically all of the S<sup>35</sup> passed through the cellophane membrane. After 2 mg, however, 52.0 per cent was non-dialyzable. This latter finding appeared at first to be contradictory to the data in Table I, Experiments 12 and 13, in which no difference was

TABLE II

*Fraction of S<sup>35</sup> in Rat Urines Which Is Non-Dialyzable after Intraperitoneal Injection of Small Amounts of Sodium Chondroitin Sulfate-S<sup>35</sup>*

Urine was collected for 24 hours following the administration of 1 mg of the S<sup>35</sup>-labeled chondroitin sulfate to one rat and 2 mg to another. Duplicate portions of each urine were dialyzed against water in rocking dialyzers for 24 hours at 0°

Chondroitin sulfate-S <sup>35</sup> injected	Non-dialyzable S <sup>35</sup> in urine
mg	per cent
1	1.07
2	52.13

TABLE III

*Coprecipitation of Chondroitin Sulfate with Barium Sulfate*

Sodium chondroitin sulfate-S<sup>35</sup> ( $1.83 \times 10^4$  c.p.m. per mg) was added to diluted rat urine. Immediately thereafter and 24 hours later portions of the urine were taken for analysis. 1 hour after the addition of 5 ml of a 0.05 N solution of sodium sulfate, 1 ml of a 2.5 N solution of hydrochloric acid, and 5 ml of a 10 per cent solution of barium chloride to each portion of urine, the barium sulfate was isolated by filtration onto paper disks for counting. The total S<sup>35</sup> added to the urine was determined similarly after extensive acid hydrolysis.

Sodium chondroitin sulfate-S <sup>35</sup> added to urine	S <sup>35</sup> isolated in inorganic sulfate	
	Immediately	After 24 hrs *
mg	per cent	per cent
1	82.3	61.8
2	53.4	55.5
3	39.4	52.8

\* The samples were held at 20° for 24 hours. During this time the pH changed from 7.3 to 8.7.

found between the specific activities of the sulfur in the inorganic sulfate and total sulfate fractions when as much as 5 mg of the chondroitin sulfate-S<sup>35</sup> were injected. An explanation of the apparent discrepancy was found in experiments in which inorganic sulfate was precipitated as barium sulfate from urines to which chondroitin sulfate-S<sup>35</sup> had been added (Table III). Though the chondroitin sulfate-S<sup>35</sup> in the absence of inorganic sulfate did not precipitate as the barium salt, it is clear that, depending upon

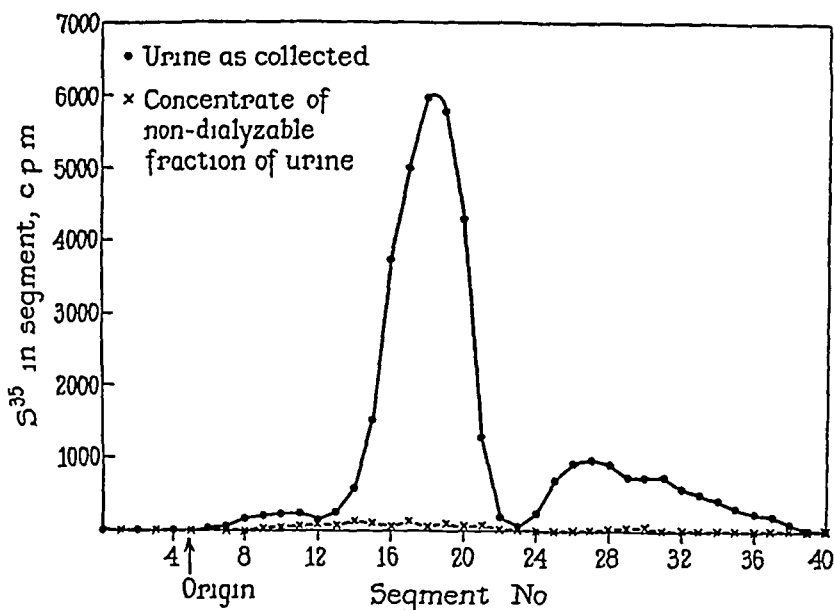


FIG 1 Electrophoretic analyses of  $S^{35}$ -labeled components in the urine of a rat given sodium sulfate- $S^{35}$  ( $100 \mu c$ ) by intraperitoneal injection. The urine was collected during the following 17 hours. For analysis of the undialyzed urine one-twenty-fifth of the total volume was used, whereas in the case of the non-dialyzable fraction of the urine the amount used was equivalent to eighteen-twenty-fifths of the total. Barbitol buffer, pH 8.6,  $\mu$  0.1, was used with a starch block that was 1 cm thick, 5 cm wide, and 62 cm long. The separation of the  $S^{35}$ -labeled components as shown was achieved in 5 hours at 12 ma in a cold room at  $0^\circ$ . Segments 1 cm wide were each eluted with 2 ml of a 1 per cent sodium chloride solution, 1 ml portions were subsequently dried for analysis.

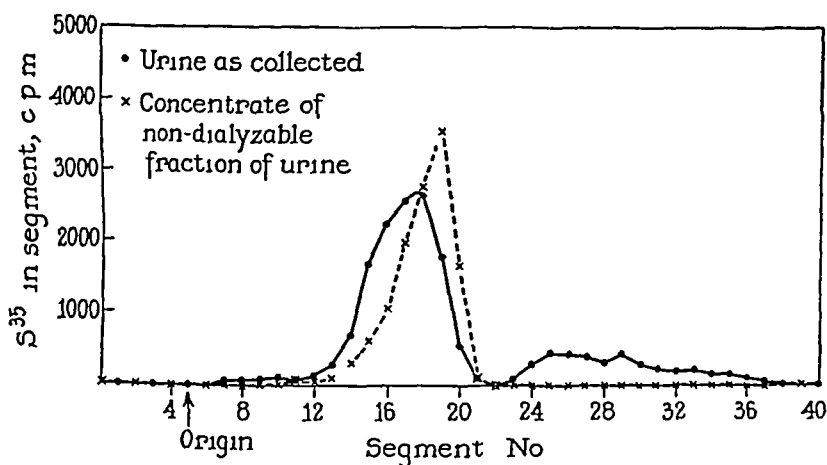


FIG 2 Electrophoretic analyses of  $S^{35}$ -labeled components in the urine of a rat given 15 mg of sodium chondroitin sulfate- $S^{35}$  ( $2.75 \times 10^5$  cpm) by intraperitoneal injection. The urine was collected during the following 17 hours. For analysis one-tenth of the total volume was used in the case of the undialyzed urine, in the case of the non-dialyzable fraction of the urine the amount was equivalent to one-fifth of the total volume. Otherwise the analyses were the same as those in Fig 1.

the amount present, a large or small fraction of it will either coprecipitate with or be occluded in barium sulfate during the precipitation of the latter. As a result it is likely that, in Experiments 12 and 13, if unchanged chondroitin sulfate was excreted in the urines, the specific activity of the sulfur in the inorganic sulfate fraction would appear to be as high as that of the sulfur in the total sulfate fraction.

So far the data only suggested the possible excretion of unchanged chondroitin sulfate or of some fragment (or fragments) of it with the sulfate still attached if the dose were 2 mg or more. Convincing proof that some chondroitin sulfate was excreted unchanged was obtained by the use of paper chromatography and electrophoresis on paper and in a starch block.

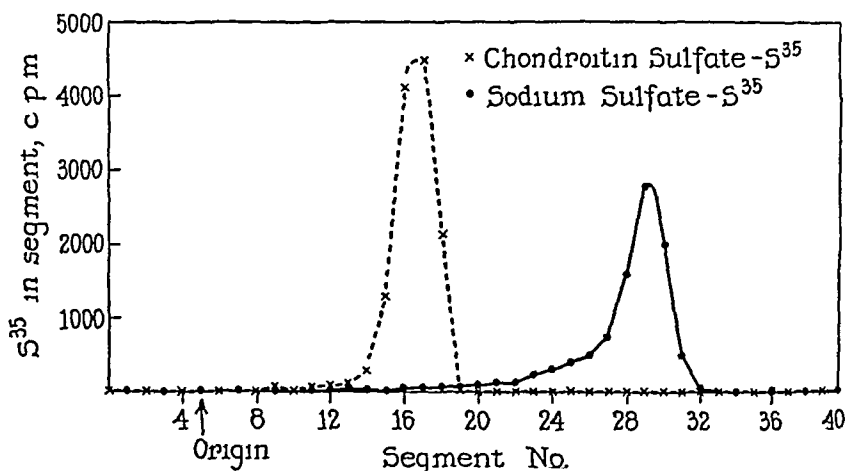


FIG 3 Electrophoretic analysis in a starch block of the sodium sulfate- $S^{35}$  and sodium chondroitin sulfate- $S^{35}$  administered to the rats whose urines were used to obtain the patterns shown in Figs 1 and 2, respectively. Analysis as given in Fig 1.

For example, in the case of a urine from a rat that received sodium sulfate  $S^{35}$  and another from a rat given chondroitin sulfate- $S^{35}$  intraperitoneally, similar patterns were found on electrophoretic analysis by use of starch blocks (Figs 1 and 2). In each of the urines there was  $S^{35}$ -labeled material that moved as did inorganic sulfate (cf Fig 3) and, in addition, materials with a slower mobility. Concentrates of the non-dialyzable fraction of the urines, however, gave dissimilar patterns. Practically all of the  $S^{35}$  in the urine from the rat that received labeled sodium sulfate passed through the cellophane membrane (Fig 1). In the concentrate of the non-dialyzable fraction of the urine from the rat which had been injected with chondroitin sulfate- $S^{35}$  there was material with a mobility like that of the administered chondroitin sulfate- $S^{35}$  (Figs 2 and 3). Electrophoresis on paper and paper chromatography (Fig 4), also indicate this. By using electrophoresis on paper, material akin to chondroitin sulfate was

detected in concentrates of dialyzed urine even when as little as 5 mg of chondroitin sulfate- $S^{35}$  had been injected

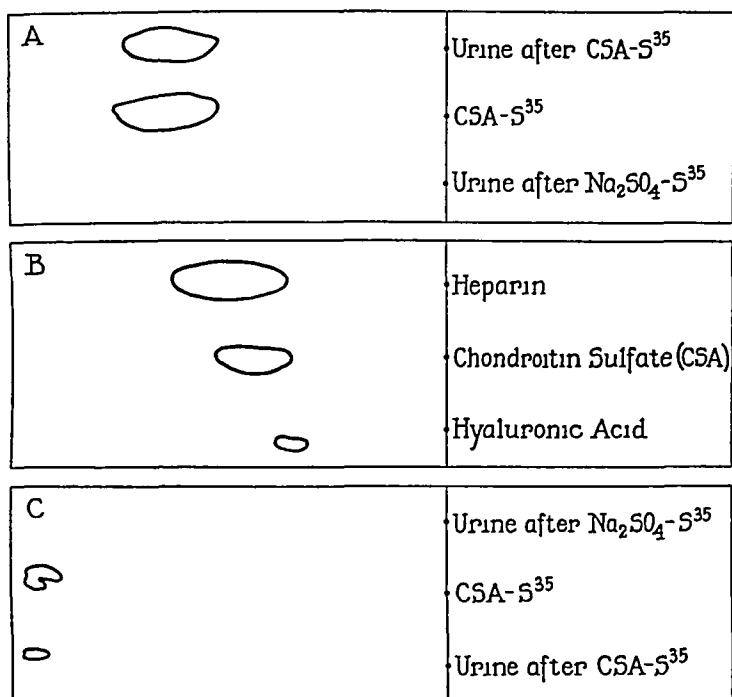


FIG 4 A, electrophoretic analyses of materials in concentrates of the non-dialyzable fraction of the urines from rats given intraperitoneally 15 mg of sodium chondroitin sulfate- $S^{35}$  and sodium sulfate- $S^{35}$  (100  $\mu$ c). In each instance an amount equivalent to one-two hundred and fiftieth of the original urine volume was used. Approximately 10  $\gamma$  of sodium chondroitin sulfate- $S^{35}$  in 0.01 ml of buffer were applied to the paper for reference. Acetate buffer, pH 4.3,  $\mu$  0.1, was used in conjunction with Whatman No. 3MM filter paper strips 3.7  $\times$  14 inches, 1 ma of current was allowed to flow for 17.5 hours at 0°. The papers were then dipped into a 0.1 per cent solution of toluidine blue in 30 per cent ethanol. Excess dye was removed by repeated washing in 30 per cent ethanol containing 0.5 per cent acetic acid. B, the possible separation of chondroitin sulfate from heparin and hyaluronic acid is apparent. Analysis as in (A) except that the running time was 16 hours. 5  $\gamma$  of heparin and 10  $\gamma$  of chondroitin sulfate and hyaluronic acid (umbilical cord) were applied to the paper, each in 0.01 ml of water. C, paper chromatogram, prepared according to Kerby (28), of the materials used in (A). The amounts applied to the paper were also the same as those in (A).

A consideration of all the data leads to the conclusion that the intact adult rat can split the bond holding the sulfate in chondroitin sulfate. The rat's capacity to do so is limited when chondroitin sulfate isolated from the skeletons of rats is injected intraperitoneally. The injection of 5 mg or more of such material results in the urinary excretion of some of



it unchanged. It is probable that the chondroitin sulfate had been depolymerized in the process of isolation to give fragments of a size which could be excreted by the kidneys.

#### SUMMARY

Chondroitin sulfate-S<sup>35</sup> isolated from the skeletons of suckling rats that had received sodium sulfate-S<sup>35</sup> was injected intraperitoneally into or fed by stomach tube to adult rats. After tube feeding no difference was found between the specific activity of the urinary inorganic sulfate sulfur and the specific activity of the urinary total sulfate sulfur, even when the dose was 20 mg of chondroitin sulfate-S<sup>35</sup> per rat. On the other hand, after intraperitoneal injection an equivalence of the specific activities of the sulfur in these two fractions was found only when the dose was 5 mg or less. After the intraperitoneal injection of 2 mg or more of chondroitin sulfate-S<sup>35</sup>, some of the S<sup>35</sup> excreted in the urine was non-dialyzable. In concentrates of the non-dialyzable fractions of these urines material was found which on electrophoretic analysis and by paper chromatography resembled the chondroitin sulfate-S<sup>35</sup> which had been injected.

It is concluded that the rat is capable of severing the ester linkage between sulfate and carbohydrate in chondroitin sulfate.

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# THE METABOLIC FATE OF RUTIN AND QUERCETIN IN THE ANIMAL BODY

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The finding of 3,4-dihydroxyphenylacetic acid (DHPAA) in the urine of rabbits after the oral ingestion of rutin or its aglycone quercetin has been reported from this Laboratory (1). In this same report it was stated that in each case after the administration of the flavonoid an examination of paper chromatograms of the ether extracts of the urine revealed the excretion of at least four metabolites (one of which was DHPAA). Two more of these four substances have now been identified (2) and a more detailed report is presented herein.

One of these two compounds, *m*-hydroxyphenylacetic acid (mHPAA), was isolated and identified in this Laboratory over a year ago, but publication was withheld pending identification of the second compound, 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid). In the meantime, at least two other laboratories have reported the finding of mHPAA in control urines (3, 4). Also included in the present report are the results obtained from three species besides the rabbit, namely rats, guinea pigs, and humans. The metabolic fate of a flavonoid such as quercetin in animals other than the rabbit was considered especially important in view of the results obtained by Clark and MacKay with rats and humans (5).

## EXPERIMENTAL

All urines were collected in receiving flasks containing sufficient hydrochloric acid to maintain an acid pH in order to retard oxidation of labile phenolic excretory products. The acid urines were extracted for 5 to 6 hours with ether in a continuous liquid-liquid extractor, and the ether extracts were evaporated to dryness under vacuum and nitrogen. Chromatograms were prepared by dissolving the ether residues in a few ml of acetone, air-drying a fraction of the acetone solution (0.02 to 0.10 ml) on a sheet of Whatman No. 1 filter paper, and migrating the fraction in two dimensions. The solvent for the ascending migration in the first direction (16 hours) was the lower phase of a mixture of chloroform, acetic acid, and water (2:1:1 by volume). Aqueous potassium chloride (20 per cent) was the solvent for the ascending migration in the second direction (2 to 3

hours) The solvent fronts were allowed to advance 30 to 35 cm in each direction. Detection of the location of the various hydroxyaromatic substances on the air-dried papers was accomplished by spraying with a solution of freshly prepared diazotized sulfanilic acid followed by 20 per cent sodium carbonate as described by Bray *et al* (6)

The mHPAA was isolated from the ether extracts of the urine of rabbits which had received quercetin (0.5 gm per kilo of body weight) via stomach tube. The basal diet was a commercial pellet preparation of natural feed stuffs. Solvent partition between ether and petroleum ether (Skellysolve F, b p 30–60°) was used to isolate mHPAA. Unfortunately, it was contaminated with small amounts of DHPAA and *m*-hydroxybenzoic acid (mHBA). Purification was achieved by preparation of the tribromo derivative, which was used to confirm the identity of the urinary metabolite as mHPAA.

Homovanillic acid was isolated from the ether extracts of urine of rats fed quercetin (2 per cent) in a diet consisting mainly of natural feedstuffs. After evaporation of the ether, the solids were extracted with hot xylene. Plates of impure homovanillic acid (HVA) were obtained as the solution cooled. These were purified by partitioning between water and chloroform, by taking advantage of the fact that homovanillic acid is more soluble in chloroform than is DHPAA.

An approximation of the quantities of the three metabolites of quercetin in urine was accomplished by migrating known quantities of the pure compounds (2 to 10  $\gamma$ ) and making comparisons of the spot size and color on paper chromatograms developed after migrating appropriate dilutions of the urine of rabbits which had received quercetin.

Since rutin and its aglycone quercetin yield the same metabolic products, quercetin was used in most of the experiments because more flavonoid could be administered per unit weight of material. The possibility of conjugated degradation products of quercetin being excreted in the urine was investigated by hydrolyzing the ether-extracted urine with 10 per cent hydrochloric acid for 3 hours, followed by ether extraction and development of chromatograms of the ether extracts.

### Results

A typical schematic diagram of a chromatogram of an ether extract of rabbit urine is shown in Fig 1. Before quercetin was given, the two most prominent spots visualized after spraying were a yellow area (Spot 4) due to mHBA and a yellowish orange spot indicating the presence of mHPAA and *p*-hydroxyphenylacetic acid (pHPAA) which migrated to the same area (Spot 2) under these conditions. However, pure mHPAA and pHPAA yield yellow and red dyes, respectively, after spraying with diazo

tized sulfanilic acid. In the case of control rabbit urine, mHPAA predominated and hence a yellowish orange spot on the chromatogram resulted.

When urine from rabbits receiving quercetin was examined chromatographically, there were at least three striking changes in evidence compared to the control. In the first place, a black area (initially red after development of the color) was located at Spot 1 in Fig. 1 and has already been

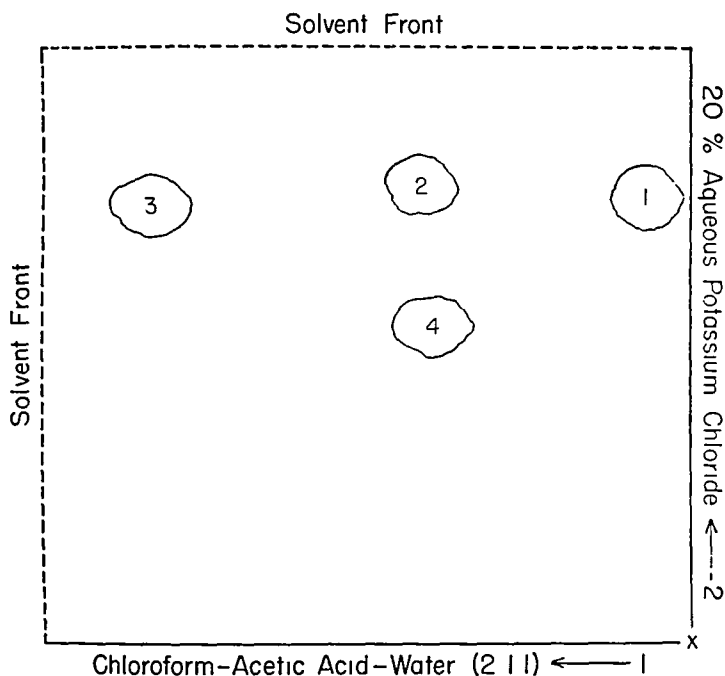


FIG. 1 Phenolic acids in rabbit urine before and after quercetin. Spot 1 = 3,4-dihydroxyphenylacetic acid, Spot 2 = *m*- and *p*-hydroxyphenylacetic acid, Spot 3 = 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid), Spot 4 = *m*-hydroxybenzoic acid.

identified and reported as being DHPAA (1). Secondly, there was a marked increase not only in the size of Spot 2 but an intensification of the yellow color characteristic of mHPAA. Thirdly, a new area (Spot 3) was noted which was red in color after spraying with diazotized sulfanilic acid and has been identified as HVA.

Paper chromatographic analyses of the urines of rats, rabbits, guinea pigs, and humans receiving oral dosages of quercetin revealed that in all four of these species a striking similarity existed in the metabolic fate of this flavonoid. In every case there was unmistakable evidence of DHPAA, mHPAA, and HVA being excreted.

Evidence has also been obtained for the urinary excretion of a substance,

or substances, that fluoresce intensely yellow in ultraviolet light and are only slightly soluble in ether (the major portion being retained in the aqueous phase after ether extraction) Work is currently in progress to isolate this material

In addition to the two-dimensional paper chromatographic criterion for the identity of mHPAA, the x-ray diffraction pattern of the tribromo derivative of the compound isolated from urine after quercetin administration has been found to be identical with that of the tribromo derivative of authentic mHPAA (Fig 2) A third criterion establishing the identity of this substance isolated from urine was the set of optical and crystallographic data which were as follows Some of the crystals of the sublimed tribromo derivative of mHPAA were plates or prisms having an end angle

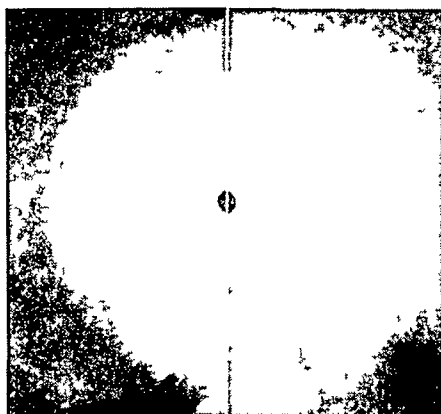


FIG 2 X-ray diffraction patterns Left side, tribromo derivative of compound isolated from urine Right side, tribromo derivative of synthetic mHPAA

of  $135^\circ$  Their birefringence was high and their extinction was sharp and symmetrical if the crystal was properly oriented Such symmetrically extinguishing side views gave the refractive index  $\gamma$  for crosswise vibrations and  $\alpha'$  for lengthwise vibrations End views (which did not extinguish sharply) showed a rhomb-shaped silhouette whose acute angle was about  $46^\circ$  The refractive indices shown by end views were  $\gamma$  for the direction which bisects the acute angle and  $\beta'$  for the other direction on those crystals oriented to give symmetrical extinction Edge views (which had a rectangular outline) showed oblique extinction with the vibration direction  $\alpha$  making an angle of  $20^\circ$  with the length of the crystal The other index obtainable from this view was  $\beta$  Acute bisectrix interference figures were sometimes obtainable from suitably tilted end views They showed that the optical character is negative with  $2E = \text{medium}$  ( $2E$  calculated =  $66^\circ$ ) ( $2V$  calculated =  $36^\circ$ ), dispersion ( $v > v$ ) slight The refractive indices are as follows  $\alpha = 1.534$ ,  $\alpha' = 1.558$ ,  $\beta = 1.77$ ,  $\gamma = 1.80$

Likewise, in the case of homovanillic acid, it was found that the chromatographic evidence including  $R_F$  values in two dimensions plus the quality of the color with diazotized sulfanilic acid was identical when compared with an authentic sample of homovanillic acid. The crystallographic data have also been found to be identical and will be reported in detail in a subsequent paper.

Having established the identity of three substances found in urine after giving an animal quercetin, and taking into account their structural relationships to quercetin and to each other, we concluded that DHPAA

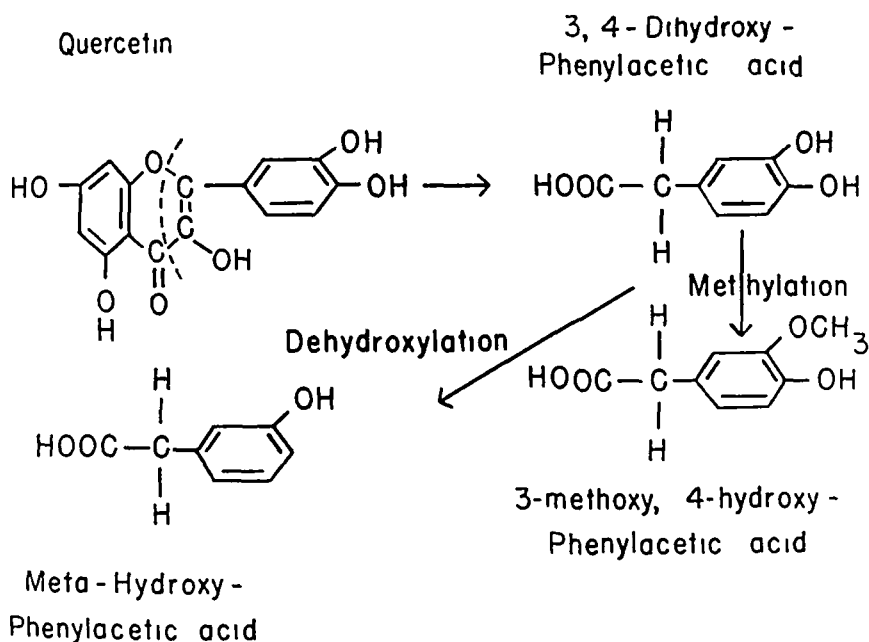


FIG 3 A proposed scheme for quercetin degradation

was probably an intermediate metabolite of quercetin and that some of the DHPAA can serve as a precursor of mHPAA by dehydroxylation and as a precursor of homovanillic acid by methylation. DHPAA was prepared by demethylation of the corresponding dimethoxy derivative and given orally to both rats and rabbits. As predicted, chromatograms were the same as those after the administration of quercetin. In other words, the administration of DHPAA resulted in the excretion of unchanged DHPAA, and there was unmistakable evidence of an increase in mHPAA as well as a definite area corresponding to homovanillic acid. Based on these results the proposed pathway of quercetin degradation in the animal body is shown in Fig 3.

Another point which was briefly investigated was the question as to whether quercetin degradation takes place in the intestinal tract or in the



animal tissues after absorption from the gut. When quercetin was administered by intraperitoneal injection, in order to eliminate possible effects due to digestive enzymes or intestinal bacteria, unmistakable chromatographic evidence of homovanillic acid was found in the urine, as was the case when it was given orally. At the present time we have no explanation for the failure to find DHPAA or an increase in mHPAA. An analysis (chromatographic) of the intestinal contents of rats receiving quercetin in the diet was negative in regard to the presence of quercetin metabolites found in the urine.

Paper chromatograms of ether extracts of hydrolyzed urines failed to reveal any additional metabolites of quercetin which might have been conjugated.

Having identified three metabolites of quercetin in the urine, we attempted to estimate the amount of these compounds being excreted when a given dosage of quercetin was administered. When 2 gm of quercetin were given to a rabbit, the values were 150 to 200 mg of DHPAA, 20 to 40 mg of homovanillic acid, and 25 to 45 mg of mHPAA (corrected for a normal excretion of 10 to 20 mg per 24 hours).

#### DISCUSSION

As an outgrowth of this work, a most interesting finding was the ability of the animal either to methylate a "meta" hydroxyl group or to remove a "para" hydroxyl group from a phenolic acid (DHPAA). This will be treated in more detail in a subsequent paper on the metabolism of 3,4-dihydroxyphenylalanine.

To date, all of the metabolic products which have been identified appear to have originated from that portion of the quercetin molecule containing the catechol nucleus (Fig. 3). The opposite side of the quercetin molecule has not as yet been accounted for. The possibility of using radioactive carbon to investigate this phase of the problem presents itself.

These results indicate that quercetin is the precursor of three urinary metabolites, two of which may be derived from DHPAA. Then it follows that a considerable amount of the orally administered quercetin must have been absorbed from the gastrointestinal tract. In view of the widespread occurrence of various flavonoids in the plant kingdom (7), it is not unreasonable to suspect that numerous flavonoids are continuously being ingested by animals, including man, and probably account for some of the many phenolic acids found in normal urines. Bray and Thorpe (8) have reviewed the results reported by various workers on the excretion of phenolic acids in the urine of rabbits on natural diets. They mention the possible role of the diet as a source of precursors of the thirty or more phenolic excretory products found in the urine, many of which have been

identified. However, they did not specifically implicate plant pigments including flavonoids as possible precursors of these urinary metabolites. These workers included pHPAA but not mHPAA in their list of urinary metabolites. Perhaps our use of a different basal diet than the one they used accounts for what might appear to be conflicting results. The use of purified diets in subsequent investigations should also aid in establishing a relationship, if any, between dietary constituents and urinary metabolites.

In a recent review by Willaman (9) on the biological effects of flavonoids, a summary of the data revealed that no less than thirty-three different types of physiological and biochemical activities have been reported for one or another of thirty different flavonoids. A question as yet unanswered is whether the degradation products of quercetin can exert the same physiological effects as those attributed to quercetin itself.

#### SUMMARY

Evidence has been presented which permits the identification of two additional metabolites in the urine after the animals were given rutin or quercetin orally, namely *m*-hydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid. 3,4-Dihydroxyphenylacetic acid, also a metabolite of quercetin, has been shown to be the precursor of the two metabolites first mentioned.

The identification of these urinary metabolites of a flavonoid such as quercetin is offered as evidence of intestinal absorption having taken place.

The urinary excretion of *m*-hydroxyphenylacetic acid after feeding quercetin implicates flavonoids in the diet as being at least one example of a dietary precursor of a *m*-hydroxyaromatic acid in urine.

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# THE NATURE OF LABILE CITROVORUM FACTOR IN HUMAN URINE

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The occurrence in human urine of a factor labile to heat and alkali which replaces citrovorum factor (CF, 5-formyl-5,6,7,8-tetrahydrofolic acid, 5-formyl-THFA) in supporting the growth of *Leuconostoc citrovorum*, strain 8081 (*Pediococcus* sp (1)) has been observed (2). This growth activity is lost upon autoclaving (pH 6.0 to 7.0) or during storage of urine at room temperature at pH 4.5 or above. After ingestion of a test dose of folic acid (FA), the bulk of the activity excreted in the urine consisted of an unstable form, and only 5 to 10 per cent of the total activity was in the form of CF. Similar products, which are unstable to heat and support the growth of *L. citrovorum*, have been observed in bacterial cell preparations by Nichol *et al* (3) and Wacker *et al* (4) and in liver enzyme reaction mixtures by Deodhar *et al* (5). We now wish to describe (a) a procedure for the separation of this labile compound in a highly purified state and (b) its properties (stability, biological, spectrophotometric), which are identical with those of anhydrocitrovorum factor (the acid degradation product of folic acid of May *et al* (6) and anhydroleucovorin of Cosulich *et al* (7)).

## Materials and Methods

The assay procedures which employ *L. citrovorum* 8081 and *Streptococcus faecalis* R 8043 (SFR) as test organisms have been described (2, 8). They were modified in that the diluted samples were added to the assay tubes at levels of 0.2, 0.4, 0.6, 0.8, and 1.0 ml. Unless otherwise noted, all the samples were autoclaved, prior to assay, at 10 pounds for 30 minutes in the presence of ascorbic acid (50 mg in a total volume of 1.5 ml, pH 6.2). This treatment converts "labile" CF to CF, the 5-formyl-THFA compound.

The urine used in this study was obtained from normal adult males who had ingested 125 mg of FA (Folvite Lederle) and 1 gm of ascorbic acid. The urine was collected and stored at once at  $-10^{\circ}$ . The subjects in general excreted 75 to 90 per cent of the FA ingested as material active for SFR and 0.5 to 3.5 per cent as substances active for *L. citrovorum*.

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Anhydroleucovoim was prepared by acidification of Ca leucovoim with HCl (6, 7). This material was then chromatographed on Dowex 1-formate, eluted with 2 per cent formic acid, and lyophilized. Since the starting material (leucovoim) consisted of a mixture of 2 diastereomeric forms, only one of which is active, the anhydroleucovoim, according to weight, should be half as active as CF. The lyophilized product, after being autoclaved in ascorbate solution, was 47 per cent as active as Ca CF for *L. citrovorum*.

Absorption measurements in the ultraviolet region were carried out in a Beckman model DU spectrophotometer.

Studies on the animal activity of the isolated compound were made with 10 day-old chicks fed a folic acid-deficient ration described by Biggs *et al* (9).

#### EXPERIMENTAL

*Purification Procedure*—After the frozen urine was partially thawed, the pH was adjusted to 2.5 to 2.6 with concentrated HCl, and the thawing was continued overnight at room temperature. The urine, still containing ice particles, was then readjusted to pH 2.5 with additional concentrated HCl. From 2 to 5 liters of the adjusted urine were filtered through Eaton and Dikeman No. 541 fluted filter paper (33 cm diameter) at 0–5°. The filtrate was discarded. The insoluble fraction was suspended in 3.5 liters of water at 0°, again adjusted to pH 2.5 with 2 N HCl, and stirred (Magne stir) 15 minutes at 0°. The suspension was filtered through a bed of Hyflo Super-Cel, and the insoluble residues were rejected. 0.5 to 1 gm of Darco-KB was added to the filtrate (first extract) and stirred (Magne stir) for 15 minutes at 0°. The Darco-KB, containing the adsorbed labile compound, was recovered by filtration on a Buchner funnel and the adsorbate stored in a freezer until used.

An example of the recovery of biological activity effected under these conditions is shown in Table I. Between 20 and 25 per cent remained in the filtrate, and 70 to 75 per cent of the activity calculated to be present in the precipitate was recovered in one extraction with water. Approximately 10 per cent more can be recovered in a second extraction. Essentially all the activity in the extract can be adsorbed on Darco-KB. The distribution of activity due to CF as such is also shown (see the values in parentheses). About 60 per cent of this 5-formyl derivative was left in the filtrate, and less than 2 per cent of the total CF activity in the extract was due to the 5-formyl derivative as such. From this point on, the contribution of CF to the total CF activity was 2 per cent or less, the remainder consisting of labile material.

The eluent used to remove the "labile" material from the Darco-KB adsorbates consisted of 70 per cent ethanol containing 2 mg per ml of K

ascorbate at pH 6.2 The frozen adsorbate was transferred with 150 ml of chilled eluent to a beaker in an ice bath For the first elution, the pH of the suspension was adjusted to 7.0 to 8.0 with 28 per cent  $\text{NH}_3$  solution under a stream of helium and stirred for 5 minutes at  $0^\circ$  The Darco-KB was recovered by centrifugation (5 minutes,  $5^\circ$  at 3000 r p m) and the eluate under helium was adjusted to approximately pH 3.0 with 2 N HCl The Darco adsorbate was eluted an additional four to five times as described above However, in these elutions, the Darco suspensions were all adjusted to pH 10.0 to 10.5 with 28 per cent  $\text{NH}_3$  solution All the eluates were stored at  $-10^\circ$  for further use

An example of the results from one of the more successful elution operations is shown in Table II In general, the recovery of the active material

TABLE I  
*Distribution of CF Activity in Urine at pH 2.5 and 5°*

	CF activity*
	$\gamma$
Whole urine, 2700 ml	6600 (695)
Filtrate (discarded), 2700 ml	1520 (408)
Ppt (calculated)	5080
1st extract of ppt, 3500 ml	3700 (67)
" " after adsorption with 500 mg Darco-KB	128

\* Sum of labile CF and 5-formyl-THFA

The values in parentheses indicate CF activity due to 5-formyl-THFA For these determinations, the samples of filtrate and extract were adjusted to pH 6.0 to 8.0 immediately after filtering and were not subjected to preliminary treatment with ascorbic acid See the text for further details

from the Darco was not predictable and ranged from 30 to 100 per cent In practice, the first eluate was discarded because it contained relatively larger amounts of inactive material than the subsequent ones

Darco eluates containing the major portion of the "labile" CF activity were combined, concentrated by distillation *in vacuo* (20 mm of Hg, water bath  $50-55^\circ$ ) under helium The distillation was continued until the preparation was free from ethanol and contained 1 to 2 mg per ml of CF activity The concentrate was then stored in a freezer

The concentrated charcoal eluates were now subjected to chromatography on a paper pulp column (Solka-Floc SW40A) at room temperature A 100 ml burette (1.5 cm in diameter) was packed firmly to a depth of 30 cm with paper pulp suspended in water-saturated butanol 2 to 3 ml of the concentrated Darco eluates, adjusted to approximately pH 4.0 with N KOH, were absorbed on dry Solka-Floc and transferred to the column

which contained enough water-saturated butanol to immerse the sample. Two filter paper disks were floated to the top of the loosely packed sample and the sample was compressed uniformly with the aid of a glass rod. Water-saturated butanol was passed through the column until the bulk of

TABLE II  
*Elution of CF Activity from Darco-KB*

Eluate No	Ammonia added	CF activity found
	<i>ml</i>	$\gamma$
1	0.5	2,030
2	2.2	6,390
3	1.0	4,960
4	1.0	3,160
5	1.0	2,330
Recovered		18,870

4 gm of Darco-KB, calculated to contain 24 mg of labile CF activity, were eluted under conditions described in the text.

TABLE III  
*Gradient Elution of CF-Active Material from Solka-Floc Column*

Fraction No	CF activity in fraction	SFR activity in fraction	Ratio SFR CF
	$\gamma$	$\gamma$	
1			
2			
3	<50	1600	>32.0
4	<50	860	>17.2
5	130	900	6.9
6	445	800	1.8
7	480	770	1.6
8	325	453	1.4
9	165	263	1.6
10	87	163	1.9
11	50	85	1.7
12	50	55	1.1

2 mg of CF-active material were applied to the column and elution was carried out as described in the text.

dark pigments was eluted. These pigments had no CF activity and were discarded.

The active material was recovered by gradient elution. A gradient was produced by passing 20 per cent formic acid (in water-saturated butanol) into a mixing vessel sealed to the top of the burette and containing 250 ml of 0.25 per cent formic acid (in water-saturated butanol). The rate of

flow within the sealed system was adjusted to 1.5 ml per minute. 25 ml fractions were collected, sampled for assay, and stored in a freezer.

The results from one such column which are typical are shown in Table III. The SFR-active material which could represent FA or *N*-10-formyl-FA was eluted first, followed by a mixture of SFR- and CF-active compounds. In general, from 75 to 100 per cent of the starting material was recovered.

The CF-active fractions were combined and concentrated by distillation *in vacuo* under helium until all the butanol was removed. Water was

TABLE IV  
*Gradient Elution of CF-Active Material from Dowex 1-Formate*

Fraction No	CF activity in fraction	SFR activity in fraction	Ratio SFR/CF
	$\gamma$	$\gamma$	
1-4 inclusive			
5	59	67	1.1
6	92	99	1.1
7	73	70	1.0
8	165	165	1.0
9	>500	>500	
10	>500	>500	
11	260	250	1.0
12	203	203	1.0
13	98	98	1.0
14	54	58	1.1
15	43	39	0.9
16	28	30	1.1
17	18	20	1.1

Concentrated Solka-Floc cuts containing 2.25 mg of CF-active material were put on the column. Gradient elution was carried out with 20 per cent formic acid. 5 ml fractions were collected. See the text for other details.

added several times during the distillation to avoid precipitation of solids from the butanol solution during concentration. The bulk of the residual formic acid in the concentrate was removed by extraction with ethyl ether. Any residual ether was removed by distillation *in vacuo* and the sample was then stored at  $-10^{\circ}$ .

The concentrate was now chromatographed on a Dowex 1-formate column. A 50 ml burette (1.3 cm in diameter) was packed to a depth of 20 to 30 cm with Dowex 1-formate, and the column placed in a cold room ( $0-5^{\circ}$ ), where the rest of the operation was carried out. The frozen sample was thawed and adjusted to about pH 4.0 with 0.5 *N* KOH and placed on the column. Gradient elution was effected by passing 10 or 20 per



cent aqueous formic acid into a mixing vessel containing 150 ml of 0.1 per cent formic acid and then through the column. The flow rate of the sealed system was adjusted to approximately 1 ml per minute. 5 to 20 ml fractions were collected, sampled, and stored at  $-10^{\circ}$ .

An example of the results of chromatography on Dowex 1-formate is shown in Table IV. The data indicate that fractions from Dowex 1-formate were considerably improved over those obtained from Solka-Floc, in that substances active for SFR and inactive for *L. citrovorum* had been removed. There appeared to be an association between the biological activity and a yellow color present in the active samples. No spectral data

TABLE V  
*Ultraviolet-Absorbing Materials and CF Activity  
of Fractions from Dowex 1-Formate*

Fraction No	Density		CF activity per 10 ml fraction
	285 $m\mu$	360 $m\mu$	
			$\gamma$
8	0.010	0.004	<20
9	0.009	0.004	<20
10	0.027	0.016	55
11	0.126	0.190	588
12	0.170	0.308	1013
13	0.292	0.492	1575
14	0.255	0.372	1275
15	0.164	0.243	1000
16	0.160	0.184	555
17	0.321	0.114	342
18	0.504	0.070	252

Density values for 0.2 ml of sample in 3.0 ml of 0.1 N HCl

are available for the samples in Table IV. However, examination of the ultraviolet absorption spectra in 0.1 N HCl of fractions from a similar column indicated that the biologically active fractions contained material which absorbed strongly at 350 to 360  $m\mu$  (Table V). Present in varying amounts in each of these fractions was a major contaminant consisting of a material which absorbed strongly at about 285  $m\mu$ . On a dry weight basis, the more active fractions were from 20 to 50 per cent as active as Ca CF.

Active fractions from Dowex 1-formate, calculated to contain approximately 4.0 mg of CF activity, were combined and concentrated *in vacuo* to about 0.5 ml. The concentrate was then transferred to a test tube and extracted with ether to remove residual formic acid. On chilling the

residue of the ether extract, a gummy greenish yellow precipitate formed on the walls of the test tube. The supernatant liquor was separated from the precipitate by centrifugation and the residue was dissolved in 1.2 ml of 1 per cent formic acid. The precipitation procedure was repeated until there was no increase in the biological activity or change in the ultraviolet absorption pattern. The final yellow product (about 2.0 mg) obtained on lyophilization was 88 per cent as active as Ca CF.

The ratios of absorbing material at 360 and 285  $m\mu$  for the various fractions are shown in Table VI. No increase in potency or 360/285  $m\mu$  ratio was achieved after the third precipitation step. Except for the lyophilization step, the sample was maintained in an atmosphere of helium during all manipulations.

TABLE VI  
*Precipitation of Urine Product from Dowex 1-Formate Concentrate*

Starting material Dowex 1-formate concentrate	1st ppt	2nd ppt (55-60 per cent)	3rd ppt (89 per cent)	4th ppt (88 per cent)
1.1	1.31 1st supernatant solution 0.49	1.98 2nd supernatant solution 0.63	2.01 3rd supernatant solution 1.68	1.98 4th supernatant solution

The values shown indicate ratios of 360 to 285  $m\mu$  absorbing materials in fractions, and the values in parentheses indicate CF activity of lyophilized precipitates.

*Properties and Identity with Anhydrocitrovorum Factor*—The ultraviolet spectra of the product isolated from urine and of anhydroleucovorin are shown in Fig. 1. The two curves are strikingly similar and suggest that the urine product is anhydrocitrovorum factor.

Further evidence indicating a close relationship between the natural product and anhydroleucovorin is shown by the parallel absorption properties of the two products in 0.1  $N$  HCl and phosphate buffer at pH 7.0 (Fig. 2). The 350 to 360  $m\mu$  absorbing band which is absent at pH 7.0 reappears on acidification. The original intensity of absorption is not recovered after acidification, because of partial oxidation of the products which results during manipulations, despite the presence of ascorbate in the solvent.

The natural isolated material and anhydroleucovorin migrate at identical rates on paper in propanol-butanol-0.1  $N$  HCl (2:1:1), 5 per cent acetic acid in water-saturated butanol, and  $M$  formic acid in water, with the following  $R_F$  values: 0.079, 0.036, and 0.37. The latter, for  $M$  formic

acid, is that recorded by Greenberg *et al* (10) Both products under the Mineralight (model No R51) display the same type of pale blue fluorescence No fluorescent areas other than those indicated could be shown to

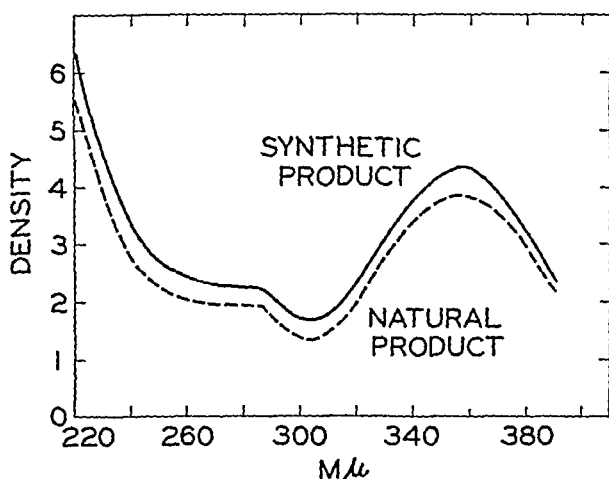


FIG 1 Ultraviolet spectra of synthetic and natural products The purity of the natural product, based on CF assay, was 88 per cent The samples, in 0.1 N HCl, contained 10  $\gamma$  of solids per ml

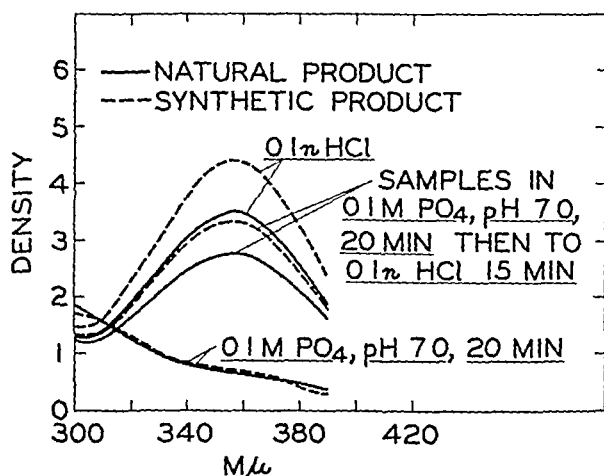


FIG 2 Spectra of synthetic and natural products in ascorbic acid (100  $\gamma$  per ml) The purity of the isolated, natural product, based on CF assay, was 77 per cent Spectra were obtained from samples containing 10  $\gamma$  of solids per ml

arise from the synthetic or natural compounds, indicating that they were essentially free of fluorescent contaminants such as free pteridines, *N*-10-formyl-FA, or *N*-10-formyl-dihydrofolic acid

Other evidence that the urine product is anhydricitrovorum factor, based on its conversion by heat, in the presence of reducing agents or absence of oxygen, to a product which is indistinguishable from CF (6, 7)

After being autoclaved in ascorbic acid, pH 6.2, the natural product gives rise to a compound which is heat-stable (*i.e.* to autoclaving at pH 6.0 to 8.0), which gives a typical growth response curve with SFR and *L. citrovorum*, and which, after storage in acid (0.1 N HCl, 1 hour, 23° in air) and autoclaving in the basal medium at pH 6.2, loses its activity for *L. citrovorum*. The urine product, dissolved in water and autoclaved 30 minutes in an atmosphere of helium, is converted into a substance which in 0.1 N KOH possesses an absorption spectrum between 220 and 390 m $\mu$  resembling that of CF (minimal at 240 to 245 m $\mu$ , maximal at 285 to 290 m $\mu$ ).

The properties of stability of the isolated urine product are those expected of anhydrocitrovorum factor. Upon being autoclaved in ascorbic acid solution, it was completely converted (purity of 88 per cent based on comparison with anhydroleucovorin at 360 m $\mu$ , Fig. 1) to CF with a ratio of SFR to CF activity of 1 (Table VII). When the sample was diluted in

TABLE VII  
*CF and SFR Growth Activities of Natural Product*

The values are given in micrograms per 100  $\gamma$

	Added aseptically in ascorbic acid*		Autoclaved in ascorbic acid		Autoclaved in basal medium	
	CF	SFR	CF	SFR	CF	SFR
Urine product	92	91	89	86	<2.5	65

Each value represents the average of four assays

\* Medium supplemented to contain 500  $\gamma$  of ascorbic acid per ml

ascorbic acid (5 mg per ml, pH 6.2) and added aseptically to previously sterilized media, the same results were obtained. The CF activity was lost during autoclaving in the absence of reducing agents, and a reduction in SFR activity also resulted. The latter loss is due in part at least to cleavage of the compound between the 6-methylene bridge and the amino group of *p*-aminobenzoylglutamic acid resulting in the liberation of a diazotizable aryl amine.

The natural product, when injected subcutaneously, is equivalent to FA in supporting the growth of the chick. A concentrate of the labile urine factor, calculated from its microbiological activity to contain the equivalent of 68  $\gamma$  of FA per 100  $\gamma$  of solids, was dissolved and diluted in 0.5 per cent ascorbate, pH 6.2, and its activity was compared with that of FA dissolved in the same solvent. Six separate doses of 8  $\gamma$  of active material in 0.5 ml of ascorbate solution were injected during a period of 18 days. At this time the average weight gains of the animals were as follows: control animals (ascorbate only), 42 gm; FA group, 185 gm; urine product group,

187 gm Each group<sup>1</sup> consisted of three animals, and the average weight of the chicks at the start of the trial was 71 gm These results are consistent with those reported by Jukes on the growth responses of chicks to anhydroleucovorin (11)

The product isolated possesses stability and growth properties for *L citrovorum* which are the same as those of a substance present in fresh whole urine (2) In order to obtain additional evidence that the isolated product was initially present in whole urine and not altered during isolation, chromatographic studies were undertaken A subject ingested 175 mg of FA and, to 20 ml aliquots of the voided urine, 10 mg of ascorbic acid were added and the aliquots stored in the frozen state The concentrations of CF-active materials in these specimens are shown in Table VIII The specimens were thawed, spotted on paper together with the reference com

TABLE VIII  
*Appearance of CF Activity in Urine after Ingestion of Folic Acid*

Sample	Time	Volume voided	pH	CF activity per ml	
				Autoclaved in ascorbic acid solution	Autoclaved in basal medium
	<i>hrs</i>	<i>ml</i>		$\gamma$	$\gamma$
A	3	185	5.62	0.65	0.057
B	5	340	5.70	0.58	0.055
C	7	70	5.31	5.80	0.450
D	9	85	5.40	3.10	0.290

175 mg of folic acid ingested by mouth at 0 hours

pounds, and chromatographed Acidic solvents consisting of propanol, butanol, and 0.1 N HCl or 5 per cent acetic acid in water-saturated butanol were used for development In these solvents *N*-10-formyl-THFA would migrate as anhydrocitrovorum factor Urine Samples C and D and the natural and synthetic products each showed, at the same location, a light blue fluorescent spot In all trials with these solvents, the light blue fluorescent material in urine migrated identically with the purified natural material and the synthetic sample The addition of either of these to urine Samples C or D did not give rise to an additional spot but did result in increased intensities of fluorescence If the urine specimens were first autoclaved for 10 minutes at 10 pounds, the biological activity for *L citrovorum* was reduced to the values shown in the last column of Table VII, and the characteristic light blue fluorescent spot no longer appeared in the chromatograms The spot characteristic of anhydroleucovorin did not appear when Ca leucovorin alone or Ca leucovorin added to normal urine was chromatographed in these acidic solvents

In aqueous formic acid, the whole urine specimen demonstrated no typical fluorescent areas in the anhydrocitrovorum factor zone. This was probably due to the low concentration of active material ( $<10 \gamma$  per ml). The isolated natural and synthetic products were difficult to render visual in this solvent with the relatively high concentrations employed ( $50 \gamma$  per ml).

#### DISCUSSION

The data reported are consistent with the view that the factor responsible for the bulk of the CF activity of urine has been obtained in a purified state in the form of anhydrocitrovorum factor. Under anaerobic conditions anhydrocitrovorum factor is in equilibrium with *N*-10-formyl-THFA, and the existence of the ring form is favored by high H<sup>+</sup> ion concentrations (6, 7). The ring compound in solution is the more stable of the two (it is less susceptible to oxidation). No attempt has been made to separate these two activities, but the isolation procedure has been such that any *N*-10-formyl-THFA initially present would tend to be converted to anhydrocitrovorum factor. The stability, growth, and chromatographic properties of the isolated material are in agreement with those of the active material present in freshly voided urine. It would thus appear that the material isolated is not an artifact formed in the isolation procedure.

The chemical interrelationships of the reduced and formylated derivative of FA have been described in detail by May *et al.* (6) and more recently by Nichol *et al.* (3). In view of the interrelationships of these compounds, the source of the *N*-10-formyl-FA reportedly found in urine (2) becomes apparent. Ring opening at pH 7.0 of the anhydrocitrovorum factor would give rise to *N*-10-formyl-THFA, and prolonged exposure to air would result in the formation of *N*-10-formyl-FA.

The possibility that some of the CF initially present in the urine had been converted to anhydrocitrovorum factor cannot be rigorously excluded. However, in the initial precipitation procedure at pH 2.5 and 0°, the bulk of the preformed CF was rejected in the filtrate. Therefore, quantitative considerations render unlikely the possibility that any significant fraction was derived from CF initially present (Table I).

Greenberg has shown that the 1-carbon unit of *N*-10-formyl-THFA and anhydrocitrovorum factor may be donated directly to 5-amino-4-imidazole-carboxamide-5'-phosphoriboside to form inosine 5'-phosphate. For a consideration of the significance of enzymatic reactions involved in the syntheses and transformylations of these reduced and formylated derivatives of FA, the reader is referred to the work of Greenberg (12, 13) and of Greenberg and Jaenicke (12-14).

According to the properties published, it seems likely that the products described by Nichol *et al.* (3), Wacker *et al.* (4), and Deodhar *et al.* (5) are

identical with or closely related to anhydrocitrovorum factor or *N*-10 formyl-THFA

### SUMMARY

A heat-labile material which supports the growth of *Leuconostoc citrovorum* has been separated from human urine in a highly purified state. The isolated material possesses the stability, biological, spectrophotometric, and chromatographic properties of anhydrocitrovorum factor (*N*-5 to *N*-10 bridge compound). It is concluded that the bulk of the growth supporting activity for *L. citrovorum* which occurs in human urine after a test dose of folic acid is due to anhydrocitrovorum factor and *N*-10-formyl tetrahydrofolic acid.

The authors are indebted to Dr G M Briggs and Dr M R Fox for determining the growth activity of the isolated material in the chick. They wish to thank J D Silverman for assistance given during the preparation of the charcoal adsorbates.

*Addendum*—The observations of Albrecht and Broquist (15) are in agreement with the present findings.

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# CHICKEN LIVER GLUTAMIC DEHYDROGENASE\*

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During the course of fractionation of chicken liver extracts, it was observed that glutamic dehydrogenase could be obtained in the crystalline form after a few simple purification steps. The procedure used in the isolation of glutamic dehydrogenase of chicken liver as well as some of the properties of the crystalline enzyme are described in the present communication. Although the chicken liver enzyme appears to be similar to the crystalline glutamic dehydrogenase from beef liver (1-3) previously described, the present results are reported in view of the simplicity of the isolation procedure and the availability of the starting material.

## *Materials and Methods*

The pyridine nucleotides were the products of the Sigma Chemical Company, and ribose nucleic acid, L-glutamic acid, and  $\alpha$ -ketoglutaric acid were purchased from the Nutritional Biochemicals Corporation. The reagent solutions were prepared as described by Olson and Anfinsen (1).

Enzymatic activity was determined by measuring the change of optical density of the reaction mixture at 340 m $\mu$  in a model DU Beckman spectrophotometer. The composition of the reaction mixtures is described under "Results," in all cases the reaction having been initiated by the addition of the enzyme. The amount of enzyme added to the reaction mixture was chosen so that the rate of the reaction was proportional to enzyme concentration and essentially linear for at least 5 minutes. Unless otherwise indicated, enzymatic experiments were carried out with three times crystallized glutamic dehydrogenase.

For comparative purposes, the activity of the enzyme was defined in the same manner as that described by Olson and Anfinsen (1). The activity ( $\Delta E$ ) was termed the change in optical density in 5 minutes (light path = 1 cm), while the specific activity was defined as the change in optical density in 5 minutes per mg of protein in 1 ml of reaction mixture. Protein concentrations were determined by the method of Bucher (4).

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### Results

*Purification of Enzyme*—Unless otherwise specified, all fractionation procedures were carried out at 0°. An acetone powder, prepared in the conventional manner from frozen chicken liver,<sup>1</sup> was stirred for 1 hour with 10 volumes of 0.05 M phosphate buffer, pH 7.4, and the mixture centrifuged to yield the crude extract. To each liter of extract were added 122 ml of ribose nucleic acid solution (50 gm of nucleic acid and 7.5 ml of 1 N sodium hydroxide per 100 ml) and 204 ml of 95 per cent ethanol. During the dropwise addition of the ethanol, the solution was stirred and cooled gradually to -5°. The mixture was adjusted to pH 6.75 with 2 M acetic acid, stirred for 15 minutes, and centrifuged at -5°. The precipitate was discarded. At a temperature of -5°, the supernatant fluid was adjusted to pH 6.25, stirred 15 minutes, and the precipitate collected by centrifugation. The precipitate was dissolved in a volume of water approximately one-sixth that of the crude extract. To the resulting solution was added 0.6 volume of a saturated ammonium sulfate solution (saturated at 0°), and, after adjusting the pH to 8.1 with 3.0 M ammonium hydroxide, the mixture was stirred for 30 minutes. The precipitate was collected by centrifugation at 20,000 × *g* for 15 minutes and dissolved in a minimal volume of water. Within a few minutes, crystallization of glutamic dehydrogenase occurred, as indicated by a pronounced silky sheen of the mixture when stirred. Crystallization was allowed to continue overnight and the precipitate was collected by centrifugation. Recrystallization was carried out by dissolving the crystals in 0.1 M potassium phosphate buffer, pH 7.4, at room temperature, adding 0.1 volume of saturated ammonium sulfate, and allowing the mixture to stand overnight in the cold. The enzyme crystallized as thin hexagonal plates which were readily visible in a microscope.

Occasionally, the precipitation of the enzyme during the first crystallization was incomplete. In this case, 1.0 volume of saturated ammonium sulfate was added to the supernatant fluid, the precipitated protein collected by centrifugation, and the crystallization step repeated. A summary of a typical fractionation is given in Table I.

The enzyme was completely stable for at least 6 months when kept at -15° in 0.1 M phosphate buffer, pH 7.4. In the purified state, the enzyme became rapidly inactivated if diluted in the absence of salt, and was also inactivated at pH 8.0, under conditions used for the assay. This latter inactivation was prevented by carrying out enzymatic experiments in the presence of 0.05 per cent serum albumin.

*Effect of Enzyme Concentration*—In Fig. 1 is shown the proportionality

<sup>1</sup> A number of different commercial products were used without any noticeable difference.

between the concentration of the crystalline enzyme and the oxidative deamination of glutamate by DPN as well as the reverse reaction Under the conditions described in Fig 1, legend, the specific activity of glutamic

TABLE I  
*Purification of Glutamic Dehydrogenase*

Fraction	Volume	Protein	Specific activity	Total activity
	<i>ml</i>	<i>mg per ml</i>		<i>per cent</i>
Extract	1300	32.5	3.30	100
Nucleic acid-ethanol ppt	200	37.5	15.1	81
Ammonium sulfate ppt	25	52.3	47.5	44
Crude crystals	12.0	25.0	111	24
Recrystallized once	10.0	13.6	155	
" 3 times	3.5	18.6	183	

The starting material was 155 gm of acetone-dried chicken liver The activities of the various fractions were determined at pH 8.0 and 23° The reaction mixtures, 3.0 ml in volume, contained 0.167 M Tris buffer, 0.0133 M L-glutamate,  $2.0 \times 10^{-4}$  M DPN, and 0.05 per cent serum albumin

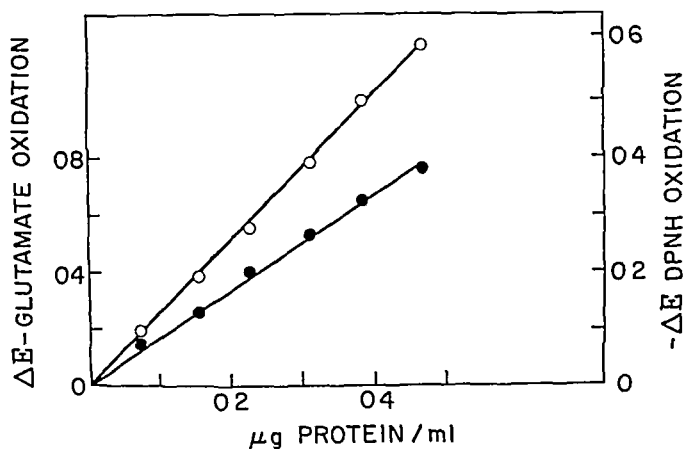


FIG 1 The effect of enzyme concentration on the initial reaction rate The experimental conditions for glutamate oxidation (●) were identical to those given in Table I Experiments in which the reverse reaction was measured (O) were carried out at pH 7.6 and 23° in the presence of 0.167 M Tris buffer,  $1.5 \times 10^{-4}$  M DPNH, 0.15 M ammonium chloride,  $1.11 \times 10^{-2}$  M  $\alpha$ -ketoglutarate, and 0.05 per cent serum albumin

dehydrogenase was 183 when the oxidation of glutamic acid by DPN<sup>2</sup> was measured and 1350 when the reverse reaction was studied When DPN

<sup>2</sup> The following abbreviations were used Tris, tris(hydroxymethyl)aminomethane, DPN, diphosphopyridine nucleotide, DPNH, reduced diphosphopyridine nucleotide

was replaced by an equimolar concentration of triphosphopyridine nucleotide, the rate of glutamic acid oxidation was one-third the rate observed with DPN

*Effect of pH*—The effect of pH on the oxidation of glutamic acid by

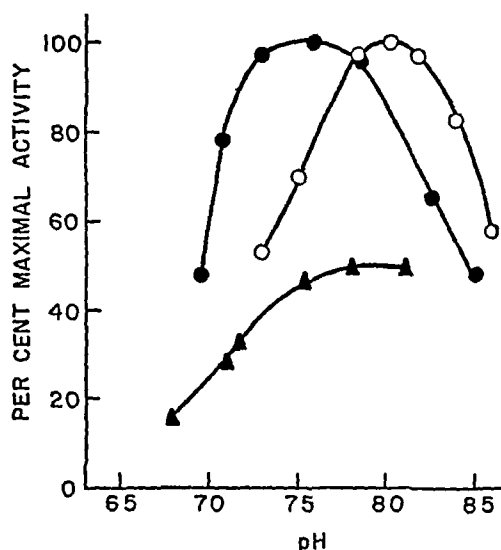


FIG 2 Effect of pH on glutamic dehydrogenase activity The experimental conditions for the oxidation of DPNH by  $\alpha$ -ketoglutarate and ammonium chloride (●) were identical to those given in Fig 1 The experimental conditions for glutamate oxidation were identical to those given in Table I except for the buffer Buffer used, (○) 0.167 M Tris, (▲) 0.167 M potassium phosphate

TABLE II  
*Inhibition of Glutamic Dehydrogenase by Salts*

Salt added	Relative activity	Salt added	Relative activity
	<i>per cent</i>		<i>per cent</i>
None	100	0.1 M $\text{Na}_2\text{HPO}_4$	77
0.1 M $\text{K}_2\text{HPO}_4$	60	0.1 M KCl	92
0.1 M $\text{Na}_2\text{SO}_4$	100	0.2 M KCl	85
0.3 M NaCl	100	0.1 M $\text{NaNO}_3$	40

The oxidation of glutamic acid by DPN was determined in the presence of the above salts under the conditions given in Table I

DPN and on the oxidation of DPNH by ammonia and  $\alpha$ -ketoglutarate is seen in Fig 2 The oxidation of glutamic acid proceeds maximally at pH 8.0 in both Tris and phosphate buffers, but the rate at pH 8.0 in phosphate buffer is only 50 per cent of that observed in Tris buffer The rate of DPNH oxidation in Tris buffer is optimal at pH 7.6 Like the oxidation of glutamic acid by DPN, the reverse reaction is also inhibited by phos

phate At pH 7.6 in Tris buffer, the rate of DPNH oxidation was inhibited 34 per cent when 0.1 M potassium phosphate was added

*Salt Inhibition*—The inhibition of glutamic dehydrogenase by a number of salts is shown in Table II. The lower activity of the enzyme in potassium phosphate buffer, as compared to that observed in Tris buffer (Fig. 2), is due to the inhibition by potassium as well as phosphate ions, since both sodium phosphate and potassium chloride are inhibitory. This inhibition

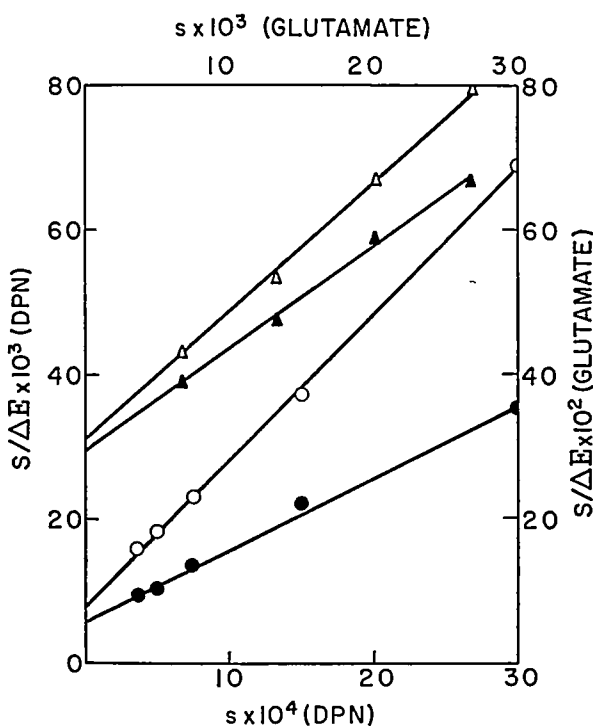


FIG. 3. Effect of substrate concentration ( $S$ ), on glutamic dehydrogenase activity in the absence (solid symbols) and presence (open symbols) of 0.1 M potassium phosphate. The experimental conditions were identical to those given in Table I, except that the concentration of DPN ( $\circ$ ,  $\bullet$ ) and glutamate ( $\Delta$ ,  $\blacktriangle$ ) were varied. The amount of enzyme present in each case was  $3.6 \times 10^{-4}$  mg per ml.

is not related to ionic strength since both sodium chloride and sodium sulfate at an ionic strength of 0.3 had no effect on enzymatic activity. The inhibition of glutamic dehydrogenase by nitrate ions is even more effective than by phosphate.

It was of interest to determine whether the inhibition by phosphate was of a competitive nature. For this purpose the effect of glutamate and DPN concentration on enzymatic activity was determined in both the presence and the absence of potassium phosphate. The results obtained were plotted according to one of the equations of Lineweaver and Burk

(5), as shown in Fig 3. If the inhibition by phosphate was competitive, it would be expected that the maximal velocity which is given by the slope of the lines plotted in Fig 3 should be independent of the presence of phosphate. The data in Fig 3 show that increasing the concentration of either glutamate or DPN does not overcome the inhibition by potassium phosphate, and therefore the inhibition is of a non-competitive type. The  $K_m$  values calculated from the data in Fig 3 were  $2.0 \times 10^{-3}$  M for glutamate, and  $6.1 \times 10^{-4}$  M for DPN.

#### DISCUSSION

The present enzyme appears to be similar to the glutamic dehydrogenase of beef liver previously described (1-3). The specific activity and pH optimum of the enzymes are approximately the same when determined under identical conditions. Both enzymes are inhibited by salts, although, under the conditions used for studying the chicken liver enzyme, the inhibition is observed at lower salt concentrations and is not related to ionic strength. The nature of the salt inhibition is not clear.

#### SUMMARY

By means of a simple fractionation procedure, glutamic dehydrogenase has been isolated in the crystalline form from extracts of acetone-dried chicken liver. The procedure is based on precipitation with nucleic acid and ammonium sulfate fractionation. The pH optimum of glutamic acid oxidation by DPN was found to be 8.0, while the reverse reaction had a pH optimum of 7.6.

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# PENTOSE METABOLISM IN WHEAT SEEDLINGS\*

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Plants contain large quantities of pentoses, chiefly xylose and L-arabinose, which exist in combined form in polysaccharides such as hemicelluloses or gums. Free pentose sugars are not encountered in plants in any significant amounts.

Comparatively little is known about pentose metabolism in plants. Earlier experiments, in which plants were infiltrated with hexose sugars, such as glucose, fructose, mannose, or galactose, showed that sucrose was synthesized *in vitro* at the expense of these monosaccharides. Since no increase of sucrose could be observed when xylose or L-arabinose was infiltrated, it was assumed that pentose sugars are not utilized for conversion into hexoses (1). However, subsequent experiments in this laboratory showed that, when  $C^{14}$ -labeled pentoses were introduced into *Canna* plants or wheat seedlings and the plants were allowed to metabolize, the sucrose isolated from the alcoholic extract was found to be radioactive, although no net increase in this disaccharide could be demonstrated during the metabolic period.

In the present work, experiments were conducted with  $C^{14}$ -labeled carbohydrates in an attempt to determine the mechanism of transformation of hexose to pentose sugars, and the reverse, pentose to hexose sugars.

## Materials and Methods

**Radioactive Sugars**—Xylose-1- $C^{14}$  was obtained from Dr. H. S. Isbell (National Bureau of Standards). Glucose-1- $C^{14}$  was prepared by an adaptation (2) of the method of Isbell *et al.* (3), and uniformly  $C^{14}$ -labeled glucose was prepared by Dr. E. W. Putman (4) in this laboratory.

Uniformly  $C^{14}$ -labeled xylose and L-arabinose were isolated from the hemicelluloses of *Canna* leaves, which had been subjected to photosynthesis for 24 hours in the presence of 50  $\mu$ c of  $C^{14}O_2$  (4). The leaves were extracted with 70 per cent ethanol, and the dried residue (1.7 gm.) was hydrolyzed with 400 ml. of 1 N sulfuric acid under a reflux condenser for 3 hours.

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Part of this paper was presented at the meeting of the Western Section, American Society of Plant Physiologists, Pasadena, June (1955).

The resulting suspension was filtered, the filtrate neutralized with barium hydroxide, the barium sulfate precipitate filtered, and the solution concentrated *in vacuo* to a syrup (0.58 gm). The syrup was taken up in 15 ml of water and applied as even bands 15 inches long on four filter paper sheets (Whatman No. 1, 17 × 22 inches) parallel to the longer edges.

In order to separate the pentose from the hexose sugars, the paper sheets were developed in a butanol-acetic acid-water mixture (5) for 48 hours, and the position of the radioactive sugars was located by exposing the dried sheets to Eastman Kodak "no screen" x-ray film. The band containing the pentoses was eluted with water and the solution was rechromatographed for 48 hours in a water-saturated phenol solution (5). Development in this solvent separated the xylose from the L-arabinose sufficiently so that the two pentoses could be eluted individually. In this manner, 130  $\mu$ c of xylose and 60  $\mu$ c of L-arabinose were isolated, both pentoses having a specific activity of approximately 2.5  $\mu$ c per mg. Determination of the  $C^{14}$  activity in the individual carbon atoms of the pentose chains (see "Degradation of sugars") showed that the activity was approximately equally distributed among all five carbon atoms of both pentoses.

*Absorption of Sugars by Wheat Seedlings*—The method used for incorporation of the radioactive sugars has been previously described (6). 3-day-old wheat seedlings were separated from the grain and placed in aerated solutions of radioactive sugars of approximately 0.1 per cent concentration. At appropriate times the seedlings were removed from the solution and the radioactive compounds formed were isolated.

*Isolation of Radioactive Carbohydrates*—The isolation of radioactive sucrose produced from the  $C^{14}$ -labeled sugars and the resolution of the disaccharide to its monosaccharide constituents have been previously described (6). The method involves paper chromatography of the ethanol extract of the seedling to isolate the sucrose, hydrolysis of the sucrose by invertase and the subsequent separation of the invert sugars by paper chromatography.

Glucose, galactose, xylose, and L-arabinose were isolated from the acid hydrolysate of the hemicellulose of the wheat seedlings as follows. In a typical experiment, the residue from 100–70 per cent ethanol-extracted seedlings (dry weight, 0.3 gm) was suspended in 20 ml of 4 per cent sodium hydroxide. The suspension was stirred with a mechanical stirrer for 24 hours at room temperature and then filtered. The residue was suspended in 5 ml of water and filtered again. The combined filtrates were acidified to pH 4.5 with concentrated hydrochloric acid and diluted with an equal volume of 95 per cent ethanol. After standing for 24 hours at 0°, the resulting crude hemicellulose precipitate was centrifuged and washed consecutively with 70 per cent ethanol, 95 per cent ethanol, and twice

with acetone The dried material (43 mg) was dissolved in 10 ml of 1 N sulfuric acid and heated in a sealed tube at 100° for 4 hours The hydrolysate was neutralized with barium hydroxide and, after filtration of the barium sulfate precipitate, the solution was concentrated *in vacuo* to a syrup The isolation of xylose and L-arabinose from this syrup was carried out as described for the isolation of the uniformly C<sup>14</sup>-labeled pentoses from *Canna* leaves The separation of four monosaccharide constituents was effected by two-dimensional paper chromatography of an aliquot of the syrup containing about 1 mg of total sugars, first in water-saturated phenol and then in butanol-acetic acid-water The separated sugars were located by exposure to x-ray film and then eluted individually It was previously shown (4, 6, 7) that radioactive sugars isolated from natural products by two-dimensional paper chromatography are devoid of significant contamination

*Degradation of Sugars*—The glucose was degraded through fermentation by *Leuconostoc mesenteroides* to CO<sub>2</sub>, ethanol, and lactic acid, a method originated by Gunsalus and Gibbs (8) The products of fermentation were further degraded chemically to their individual carbon atoms by the method of Katz *et al* (9) Details for this procedure are given in a previous publication (6)

The xylose and L-arabinose were degraded through fermentation by *Lactobacillus pentosus* to acetate and lactate by the method of Gest and Lampen (10), and the products were further degraded by chemical means (9)

*Determination of Radioactivity*—The C<sup>14</sup> activity in the barium carbonate obtained from the degradation of the sugars was estimated, 10 to 15 mg samples plated on aluminum disks being used, by means of a Berkeley decimal scaler (model No 2105) equipped with a lead-shielded, 1 inch end window Geiger tube (7) Corrections for self-absorption of the samples were obtained from a standard curve

The specific activities of the glucose, galactose, xylose, and L-arabinose were determined as follows Solutions of the sugars were obtained after two-dimensional chromatography and subsequent elution of the hemicellulose hydrolysis products The concentration of the hexoses was quantitatively determined on separate aliquots according to the method of Somogyi as modified by Nelson (11) and that of the pentoses by the method of Mejbaum (12) Aliquots of each solution were then dried on aluminum disks and counted

The amounts of C<sup>14</sup> activity in the ethanol-extractable compounds formed from uniformly C<sup>14</sup>-labeled xylose were estimated directly on two-dimensional chromatograms with a Tracerlab SU-3A rate meter supplied with a Geiger counter (10) This instrument can be read with an accuracy of  $\pm 5$  per cent of the full scale deflection in ranges of 200 to 20,000 c p m



### Results

*Absorption of Hexoses*—The distribution of  $C^{14}$  activity in the pentose sugars isolated from the hemicelluloses formed in the wheat seedlings after absorption of glucose-1- $C^{14}$  is shown in Table I, the major proportion of the activity in both pentoses residing in C-1. Significant amounts were

TABLE I  
*Per Cent Distribution of  $C^{14}$  in Hemicellulose Pentoses of Wheat Seedlings after Absorption of Glucose-1- $C^{14}$*

100 seedlings (450 mg) were incubated for 2 hours at 22° in 10 ml of medium containing 5 mg of glucose-1- $C^{14}$  with a total activity of 37  $\mu$ c. The values were obtained by *L. pentosus* degradation and are expressed as percentages of the total activity recovered as  $BaCO_3$ .

Pentose	Carbon atom No				
	1	2	3	4	5
Xylose	80.1	0.3	13.0	0.7	5.9
L-Arabinose	87.1	0.4	5.3	0.6	6.6

TABLE II  
*Comparison of Amount of  $C^{14}$  Incorporated into Hemicellulose Monosaccharides after Absorption of Uniformly  $C^{14}$ -Labeled Glucose and Glucose-1- $C^{14}$*

In both experiments, forty seedlings (180 mg) were incubated for 8 hours at 22° in 4 ml of medium containing 15 mg and 25  $\mu$ c of uniformly  $C^{14}$ -labeled glucose and glucose-1- $C^{14}$ , respectively.

Sugar absorbed	Specific activity of hemicellulose monosaccharides, $\mu$ c per mg			
	Glucose	Galactose	Xylose	L-Arabinose
Glucose-1- $C^{14}$	0.043	0.114	0.033	0.033
Uniformly $C^{14}$ -labeled glucose	0.046	0.099	0.035	0.032

also found in C-3 and in C-5, while C-2 and C-4 contained virtually no activity.

For comparison, the glucose and fructose from sucrose, and the glucose from hexose phosphate and from cellulose, were simultaneously isolated from the same seedlings, and the distribution of  $C^{14}$  activity in their carbon atoms was determined (6). In agreement with the previous results (6), C-1 of all the hexoses contained approximately 75 per cent of activity, while C-6 contained about 20 per cent.

Table II represents a comparison of the results of an experiment in which two lots of wheat seedlings were treated in an identical manner and in-

cubated with the same quantity of  $C^{14}$ -labeled glucose, with the difference that the glucose in one lot was uniformly labeled in all carbon atoms while that in the other lot was labeled only in C-1. Comparison of the specific activities of the glucose, galactose, xylose, and L-arabinose isolated from the hemicelluloses of the two lots shows that they are approximately the same whether uniformly  $C^{14}$ -labeled glucose or glucose-1- $C^{14}$  was supplied to the plants.

*Absorption of Pentoses*—Preliminary experiments with both uniformly labeled xylose and L-arabinose indicated that, like the hexoses, these pentoses were readily incorporated into sucrose and sugar phosphates. Treatment of the sucrose with invertase showed its  $C^{14}$  activity to be approximately evenly divided between the glucose and fructose moieties. Treatment of the sugar phosphates with Armour intestinal phosphatase showed their  $C^{14}$  activity to reside mainly in glucose and fructose derivatives.

The rate of incorporation of uniformly  $C^{14}$ -labeled xylose into the ethanol-extractable carbohydrates was studied as follows. Each of six lots consisting of ten seedlings was placed in 10 ml of water containing 6 mg of uniformly  $C^{14}$ -labeled xylose, with a total  $C^{14}$  activity of  $15 \mu c$  ( $1 \mu c$  equals approximately  $2 \times 10^5$  c p m by the Geiger counter used in this work). Lots were consecutively removed from the solution after 15 minutes, 1 hour, and 2 hours, and immediately extracted with ethanol. The three remaining lots were simultaneously removed from the radioactive solution at the end of 2 hours, washed thoroughly with water, and replaced in a solution of unlabeled xylose of the same concentration. These lots were removed after 1, 2, and 6 hours, and each sample upon removal was immediately extracted with ethanol. Aliquots of the ethanol extracts from all the samples were chromatographed two-dimensionally first in water-saturated phenol and then in butanol-acetic acid-water, and the activity in the ethanol-extractable compounds was estimated.

The results in Fig 1 show that the incorporation of total activity into hexose phosphates and sucrose was rapid during the absorption of  $C^{14}$ -labeled xylose. However, after the seedlings were placed in the unlabeled xylose solution, the activity in the hexose phosphates immediately decreased, while the activity in sucrose continued to increase for about an hour. The subsequent decrease in sucrose activity coincided with the appearance of  $C^{14}$  activity in the free monosaccharides, glucose and fructose.

The distribution of  $C^{14}$  activity in the carbon atoms of glucose isolated from the sucrose formed in the wheat seedlings after absorption of xylose-1- $C^{14}$  is shown in Table III. Carbon atoms 1, 3, 4, and 6 contained the major proportion of  $C^{14}$  activity.

The distribution of  $C^{14}$  in the xylose and L-arabinose of the hemicellu-

loses eliminates the hexose monophosphate shunt involving the decarboxylation of the C-1 of glucose-6-phosphate as a mechanism for their formation. If these pentoses were formed by such a mechanism, the C<sup>14</sup> would be pre-

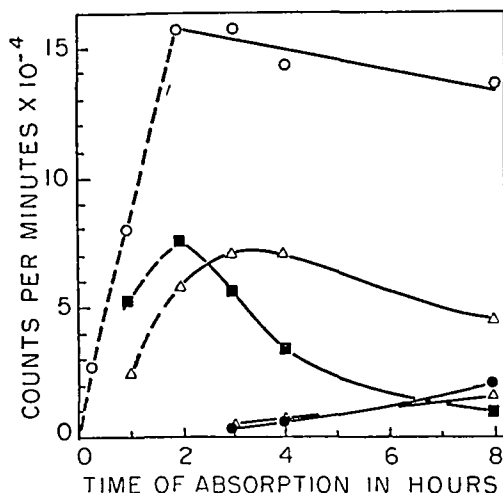


FIG 1 Incorporation of C<sup>14</sup> into the ethanol-extractable compounds of wheat seedlings by absorption of uniformly C<sup>14</sup>-labeled xylose. The dashed line represents absorption of uniformly C<sup>14</sup>-labeled xylose, solid line, absorption of unlabeled xylose, O, total C<sup>14</sup> activity in major components of ethanol extracts, ■, total C<sup>14</sup> activity in hexose phosphates, Δ, total C<sup>14</sup> activity in sucrose, ●, total C<sup>14</sup> activity in glucose, ▲, total C<sup>14</sup> activity in fructose.

TABLE III

*Per Cent Distribution of C<sup>14</sup> in Glucose Moiety of Sucrose after Absorption of Xylose-1-C<sup>14</sup>*

85 seedlings (380 mg) were incubated for 2 hours at 22° in 10 ml of medium containing 10 mg of xylose-1-C<sup>14</sup> with a total activity of 6 μc. The values were obtained by degradation of *L. mesenteroides* and are expressed as percentages of the total activity recovered as BaCO<sub>3</sub>.

	Carbon atom No					
	1	2	3	4	5	6
C <sup>14</sup> activity, %	49.1	2.0	20.5	8.6	1.0	18.8

dominantly in C-5, as the endogenous glucose-6-phosphate is labeled in C-1 and C-6 (6). This conclusion was also reached by Neish (13) from his work on the metabolism of xylose in wheat plants. The quantitative differences between his data and ours may be due to differences in the age of the plants, the duration of the experiments, or the methods of introducing the radioactive sugars.

Since the specific activities of the hemicellulose pentoses are small in comparison to the specific activity of the glucose-1- $C^{14}$  absorbed (Table II), the possibility must be considered that the  $C^{14}$  observed in these pentoses might have originated from minor pathways of pentose synthesis. This possibility is excluded by the other data in Table II which show that the specific activities of the hemicellulose hexoses and pentoses are the same after absorption of either uniformly  $C^{14}$ -labeled glucose or glucose-1- $C^{14}$ .

The fact that, after placing the wheat seedlings into unlabeled xylose solution, the activity of the hexose phosphates immediately decreased while the activity of the sucrose continued to increase (Fig 1), indicates that the initial steps in the utilization of xylose by wheat seedlings may be a conversion to hexose phosphate followed by incorporation into sucrose. The distribution of  $C^{14}$  in the glucose obtained from sucrose after the absorption of xylose-1- $C^{14}$  (Table III) is in accord with the mechanism proposed by Gibbs and Horecker (14) for the conversion of ribose-5-phosphate-1- $C^{14}$  to hexose phosphate by pea root preparations. These workers found 70 per cent of the  $C^{14}$  in the C-1 and 29 per cent in the C-3 of the hexose phosphate formed. If a similar mechanism for the conversion of pentose to hexose is active *in vivo* in wheat seedlings, this distribution would be complicated by approximately 20 per cent randomization between C-1 and C-6 and C-3 and C-4 of the hexose phosphate formed because of a reversal of glycolysis (6). This randomization would result in a  $C^{14}$  distribution that agrees closely with the observed distribution (Table III). It is possible, therefore, that the mechanism involving transaldolase and transketolase proposed by Gibbs and Horecker (14) to explain their results *in vitro* may also be operative in wheat seedlings *in vivo*.

#### SUMMARY

The interconversion of hexose and pentose was studied in actively metabolizing wheat seedlings. From the distribution of  $C^{14}$  in the carbohydrates arising from the absorbed monosaccharides, it was concluded that the conversion of hexose to xylose and L-arabinose does not involve a C-1 decarboxylation and that the conversion of xylose to hexose phosphate may involve the action of transaldolase and transketolase.

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# ENZYMATIC BASIS OF RESISTANCE TO AUREOMYCIN

## I DIFFERENCES BETWEEN FLAVOPROTEIN NITRO REDUCTASES OF SENSITIVE AND RESISTANT *ESCHERICHIA COLI*

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Nitro reductase isolated from Aureomycin-sensitive *Escherichia coli* E26 catalyzes the reduction of the nitro groups of chloramphenicol and PNB<sup>1</sup>. These reductions are inhibited by low concentrations of Aureomycin (1). Cofactor requirements for the reduction were DPN and L-malate (replaceable by DPNH), L-cysteine, and  $Mn^{++}$  (2-4). In addition, by analogy with non-bacterial systems (5, 6), a flavin was postulated as an intermediate carrier of electrons. Recently a similar enzyme with essentially the same cofactor requirements has been extracted from an Aureomycin-resistant organism derived from the parent sensitive strain. The cell-free enzyme derived from the resistant cell was, however, resistant to inhibition by Aureomycin (7). The present communication presents data indicating that both Aureomycin-sensitive and -resistant nitro reductases are flavoproteins, the conjugated flavin in the former being relatively easily dissociated, while that in the resistant enzyme is very firmly bound. In addition, data will be presented indicating that the sensitive enzyme is a metalloflavoprotein with manganese as the possible metal component. The relationship of these findings to the mode of action of Aureomycin will be discussed.

### *Materials and Methods*

Sensitive cells were grown, harvested, and extracted as previously described (2). A 5 liter culture of resistant cells was grown overnight at 37° in a flask of phosphate-buffered peptonized milk containing 60  $\gamma$  of Aureomycin per ml. This suspension was used to inoculate 45 liters of Aureomycin-free medium at pH 7.0 and the total suspension was aerated vigorously at 37° for 18 hours. Growth of the resistant organism for this

<sup>1</sup> The abbreviations used are PNB, *p*-nitrobenzoic acid, DPN, diphosphopyridine nucleotide, DPNH, reduced diphosphopyridine nucleotide, FMN, flavin mononucleotide, FMNH<sub>2</sub>, reduced flavin mononucleotide, FAD, flavin adenine dinucleotide, TCA, trichloroacetic acid.

period in the antibiotic-free medium did not lead to diminished resistance. Resistant cells were harvested and extracted exactly as the sensitive cells.

Arylamine was determined as previously described (2). DPNH oxidation and FMN reduction were followed in a Beckman DU spectrophotometer by measuring the decrease in absorption at 340 and 450 m $\mu$ , respectively. Anaerobic experiments in the spectrophotometer were carried out either in Thunberg tubes fused to 10 mm Pyrex cuvettes<sup>2</sup> or in the cuvettes designed by Lazarow and Cooperstein (8). The former cuvettes were evacuated with a Cenco-Megavac pump and addition substances were tipped in from the side arm, while the latter were made anaerobic by bubbling with nitrogen freed of oxygen by passage over copper filings heated to 400°. A black cloth was used to shield the tubes and photocell from stray light.

Free riboflavin, FMN, and FAD were determined in the Coleman photo fluorometer, model 12C, by the method of Burch *et al* (9). Conjugated flavin was extracted from the sonically disrupted cells according to Warburg and Christian (10). Crude sonic extracts were purified as described in the text. DPNH, FMN, and FAD (65 per cent) were products of the Sigma Chemical Company, St. Louis. Chloramphenicol was donated by Parke, Davis and Company, Detroit, and Aureomycin was a gift of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

*Flavin Requirement for Nitro Reductase*—In distinction to the resistant enzyme, the requirement of the sensitive enzyme for exogenous flavin became evident when extracts were fractionated by acid  $(\text{NH}_4)_2\text{SO}_4$  precipitation. An extract from sonically disrupted cells which had been dialyzed against cold, running tap water for 16 hours was centrifuged in the Spinco ultracentrifuge for 4 hours at  $144,000 \times g$ . The supernatant solution was adjusted to pH 3.0 with glacial acetic acid, then brought to 30 per cent saturation with solid  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was collected and re-suspended in 0.1 M  $\text{KHCO}_3$  and dialyzed against cold, running tap water for 18 hours. Undissolved material was centrifuged prior to use in assays. Fig. 1 shows a typical experiment in which this preparation was used. Experimental vessels containing 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.5,  $6.6 \times 10^{-5}$  M DPNH,  $5 \times 10^{-3}$  M L-cysteine,  $3 \times 10^{-4}$  M chloramphenicol, 0.5 mg of enzyme protein per ml, and various FMN concentrations in a total volume of 1.5 ml were incubated anaerobically at 37° for 2 hours. Stimulation up to 600 per cent by either FMN or FAD has been observed, riboflavin was inactive. This diastatic treatment caused

<sup>2</sup> We wish to thank Dr. M. Weber, McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland, for making available to us the design of these cuvettes.

a loss of approximately 80 per cent of the total units of enzyme, but some increase in specific activity was noted. In later experiments, the flavin requirement for the sensitive enzyme was demonstrated in the supernatant solution remaining after precipitating neutral 40 to 50 per cent  $(\text{NH}_4)_2\text{SO}_4$  fractions with 0.85 ml of 2 per cent protamine sulfate<sup>3</sup> per 100 mg of

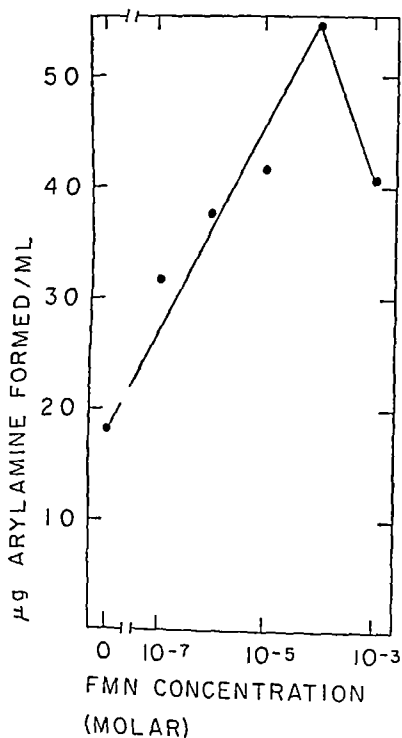


FIG 1

FIG 1 Stimulation of Aureomycin-sensitive nitro reductase by FMN

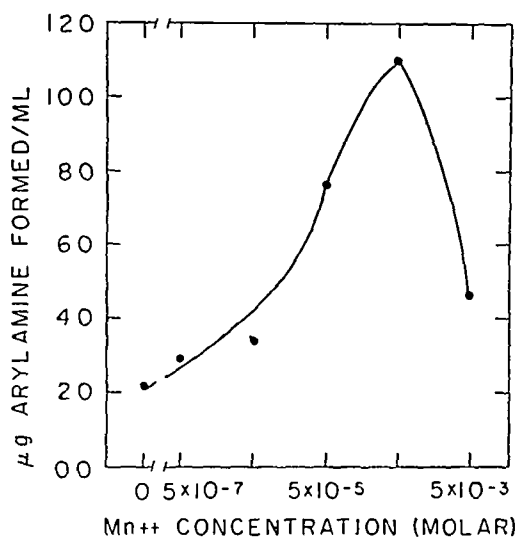


FIG 2

FIG 2 Stimulation of Aureomycin-sensitive nitro reductase by  $\text{Mn}^{++}$ . Enzyme and experimental conditions similar to those experiments reported in Fig 1 except that no FMN was added

protein. Apparently this treatment separated the reductase into its flavin component and apoenzyme.

It has not been possible to show a flavin requirement for the resistant enzyme by either of these procedures.

**Cation Requirements of Sensitive and Resistant Nitro Reductase**—It had previously been reported (3, 4) that in extracts from sensitive cells  $\text{Mn}^{++}$  was essential for arylamine formation when DPN and L-malate were used

<sup>3</sup> The protamine sulfate used in this experiment was a product of the Nutritional Biochemicals Corporation. Other commercial protamine preparations did not resolve the enzyme.



as the electron donor system. In this crude system it was postulated that the requirement for the cation was due in part to the need of the oxalacetic oxylase for  $Mn^{++}$ . (The activated carboxylase, by removing the oxalacetate formed as a result of malic dehydrogenase activity, caused a shift in equilibrium of the latter enzyme toward DPNH.) However, Fig 2 illustrates that in more resolved Aureomycin-sensitive reductase preparations, which are devoid of malic dehydrogenase activity,  $Mn^{++}$  is essential for electron transport beyond the locus of DPNH formation. Similar stimulation has been obtained when  $Mn^{++}$  is added as  $KMnO_4$ . Concentrations of  $Mn^{++}$  greater than  $5 \times 10^{-3}$  M were toxic and precipitated the enzyme preparation. In some experiments  $Mn^{++}$  and FMN produced

TABLE I  
*Flavin Content of Aureomycin-Sensitive and -Resistant Nitro Reductases*

Extract	Flavin content, $\gamma$ per mg. protein			
	Riboflavin	FAD	FMN	Total
Sensitive	Trace	0.013	0.12	0.13
Resistant	"	0.079	1.99	2.07

Both extracts were prepared by collecting 40 to 50 per cent  $(NH_4)_2SO_4$  fraction of an extract prepared by sonic disruption of cells. This fraction was then dissolved in  $H_2O$ , dialyzed, and treated with 0.85 ml. of 2 per cent protamine sulfate (Nutritional Biochemicals Corporation) per 100 mg. of protein after adjustment to pH 6.0 with dilute acetic acid. The supernatant solutions were then dialyzed overnight and treated with  $Ca_3(PO_4)_2$  gel (0.75 mg. per mg. of protein). The supernatant solutions were dialyzed for 4 hours in the cold and analyses were performed as indicated. The purification of both sensitive and resistant extracts was 22-fold.

an additive stimulation of the reductase. In certain preparations, stimulation approximately one-third to one-half that obtained with  $Mn^{++}$  was observed with  $Mg^{++}$  and  $MoO_4^{--}$ . Additive stimulation by the various cations has not been observed. On the other hand, the requirement of the resistant extract for cation is not as marked as that of the sensitive enzyme. Although arialamine formation by purified resistant preparations was stimulated 10 to 25 per cent upon addition of  $2.5 \times 10^{-3}$  M  $Na_2MoO_4$ , dialysis of the extract against triple distilled  $H_2O$ , KCN,  $NH_3$ , Versene, or other chelating agents did not result in increased requirement for the metal.

*Conjugated Flavin Content of Extracts*—With 22-fold purified extracts prepared in a precisely similar manner, the sensitive extracts were very pale yellow in color, while resistant enzyme preparations were strongly yellow. Table I indicates that the bright yellow color of the resistant

enzyme resulted from the presence of 16 times more conjugated flavin than was present in the Aureomycin-sensitive preparation. In both extracts, 90 to 95 per cent of the flavin was FMN, the remainder was FAD, with only traces of free riboflavin. The total flavin content per mg of protein increased 4- to 5-fold as the resistant enzyme was purified, while that of the sensitive enzyme increased only slightly. The absorption spectra of the two enzyme preparations and of the conjugated flavins derived from them indicated that this yellow color was primarily due to their flavin content. The resistant extract had a large peak at  $450\text{ m}\mu$ , the sensitive extract, a barely perceptible shoulder. In both extracts the flavin peaks disappeared on reduction with  $\text{Na}_2\text{S}_2\text{O}_4$  and reappeared on subsequent oxidation. It should be emphasized that the flavin of the resistant enzyme was firmly bound and resisted long periods of dialysis.

*Function of Cysteine*—Cysteine appears to function in a multiple capacity in both sensitive and resistant nitro reductase. One function of the cysteine may be related to the fact that the enzymes are presumably —SH complexes, being inhibited from 25 to 95 per cent by *p*-chloromercuribenzoate at concentrations of  $5 \times 10^{-5}$  to  $5 \times 10^{-3}$  M. This inhibition can be reversed to the extent of 75 per cent by  $5 \times 10^{-3}$  M cysteine.

It had previously been reported (1, 2) that cysteine was essential for arylamine formation from both chloramphenicol and PNB. It has now been established that the enzymatic reaction is a 2 electron reduction. In the absence of cysteine in a standard system (2) containing either sensitive or resistant enzyme, buffer, DPNH, and PNB, a non-diazotizable intermediate accumulated. This compound was shown to be *p*-nitrosobenzoic acid by the method of Feigl (11). Reduction of the intermediate with cysteine, after precipitation of the reaction mixture by TCA, resulted in the formation of a diazotizable amine. Synthetic *p*-nitrosobenzoate behaved similarly. It would thus appear that cysteine in this instance functions to reduce chemically the nitroso intermediate to a diazotizable amine.

Nevertheless, with regard to —SH requirement, certain significant differences exist between Aureomycin-sensitive and -resistant extracts (7). D-Cysteine is somewhat more effective than the L enantiomorph in promoting arylamine formation by the resistant enzyme, while the sensitive enzyme forms 2 to 3 times more arylamine in the presence of L- than in the presence of D-cysteine. Consequently, it is postulated that cysteine, in addition to its reducing capacity, functions in an as yet unknown manner in the enzymatic reaction.

*Reduction of Nitro Group by FMNH<sub>2</sub> in Sensitive Extracts*—Table II shows that DPNH can serve as a source of electrons for the reduction of FMN. The reduced flavin can then be used as an electron source for the reduction of the nitro group of chloramphenicol. Similar results were

obtained with PNB as the electron acceptor. The reaction between FMNH<sub>2</sub> and —NO<sub>2</sub> is rapid, significant amounts of arylamine being formed within 1 minute. This is in sharp distinction to the slow rate of —NO<sub>2</sub> reduction which occurs when oxidized FMN, DPNH, and the —NO<sub>2</sub> group are present together initially.

Stoichiometric amounts of DPNH are oxidized with concomitant reduction of FMN. However, the amount of FMNH<sub>2</sub> oxidized is somewhat greater than the amount of arylamine formed. The lack of stoichiometry in the latter reaction is not at present clearly understood, but may be due, at least in part, to residual amounts of O<sub>2</sub> left in the cuvettes. This O<sub>2</sub>

TABLE II  
*FMNH<sub>2</sub> As Electron Donor for Chloramphenicol Reduction*

System	Experiment 1			Experiment 2		
	DPNH oxidation	FMNH <sub>2</sub> oxidation	Aryl amine formed	DPNH oxidation	FMNH <sub>2</sub> oxidation	Aryl amine formed
	<i>μ</i> moles	<i>μ</i> moles	<i>μ</i> moles	<i>μ</i> moles	<i>μ</i> moles	<i>μ</i> moles
Complete	11.6	8.2	7.0	11.9	8.7	5.0
Without chloramphenicol	14.5	1.0	0	11.9	1.0	0

Two different enzymes were prepared as described in Table I. 1.0 ml. of enzyme (830  $\gamma$  of protein), 0.05 M phosphate buffer, pH 7.5,  $6.6 \times 10^{-5}$  M DPNH,  $1.2 \times 10^{-5}$  M FMN, and  $5 \times 10^{-3}$  M L-cysteine in a total volume of 3.0 ml. were incubated together for 30 minutes. At this time either 0.3 ml. of chloramphenicol (1000  $\gamma$  per ml.) or 0.3 ml. of H<sub>2</sub>O was tipped in from the side arm and readings were taken for 2 minutes. The reactions were run in Lazarow-Cooperstein cuvettes (8), and changes in the optical densities were followed in a Beckman DU spectrophotometer at proper wave lengths.

could conceivably reoxidize FMNH<sub>2</sub> and lead, as a consequence, to increased DPNH oxidation.

Analogous results to the above were obtained when the reduced flavin was non-enzymatically prepared by reduction with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>. Excess reducing agent was removed by bubbling with H<sub>2</sub>.

*Reduction of Nitro Group by Reduced Flavoprotein of Resistant Extracts*—When DPNH and purified Aureomycin-resistant nitro reductase were incubated together anaerobically with L-cysteine, the flavoprotein was rapidly reduced and remained so, providing a small excess of DPNH was present. Subsequent addition of chloramphenicol to the reduced enzyme caused complete reoxidation of the flavoprotein within 1 minute with the accompanying formation of stoichiometric amounts of reduced chloramphenicol.

## DISCUSSION

It is of considerable interest that the Aureomycin-sensitive and -resistant nitro reductase preparations differ so drastically in both flavin requirement and composition and in apparent cation requirement. The question arises as to whether these differences bear any relationship to the mode of action of Aureomycin. It is of course possible that the large amount of bound, conjugated flavin present in the resistant enzyme is able in some manner to reverse the inhibitory activity of the antibiotic. Though added FMN, FAD, or both together, did not reverse the inhibitory effect of Aureomycin on sensitive nitro reductase, it may be pertinent to note that riboflavin, among other compounds, has been reported to reverse the inhibition, by the tetracyclines, of the growth of various microorganisms (12, 13).

An alternative hypothesis is based upon recent observations by Mahler and Green (14) and Nicholas and Nason (6, 15). These workers have reported, respectively, that various electron transport enzymes and the nitrate reductases of *Neurospora crassa* and *E. coli* are metalloflavoproteins. Since Aureomycin is a potent chelating agent (16), and since, as the present communication indicates, there is a requirement for  $Mn^{++}$  for sensitive nitro reductase, it is conceivable that in sensitive organisms Aureomycin is active by virtue of its ability to combine with or compete successfully for essential cations of the electron transport system. The fact that the  $Mn^{++}$  requirement of sensitive nitro reductase is relatively easily demonstrated, presumably because the cation is loosely associated with protein, and the difficulty in showing even minimal cation requirement for resistant nitro reductase, presumably because the cation is very firmly bound to protein, are consistent with the above hypothesis. In this view, the relative insensitivity of resistant nitro reductase to Aureomycin could be explained by assuming that, in the development of resistance to the antibiotic, an altered protein has been synthesized which is able to bind essential cation firmly enough to compete successfully with Aureomycin. The observation that resistant enzyme contains firmly bound flavin as the prosthetic group, while sensitive enzyme possesses easily dissociated flavin, is compatible with the thesis that resistance indeed reflects the synthesis of altered enzyme.

## SUMMARY

Aureomycin-sensitive and -resistant nitro reductase concentrates were compared with respect to flavin content, flavin requirements, and cation requirements. The resistant enzyme contained firmly bound conjugated flavin (chiefly flavin mononucleotide (FMN)), while the flavin of sensitive

reductase was easily dissociable. Activation of the sensitive preparation, but not that of the resistant preparation, by  $Mn^{++}$  and by FMN was readily demonstrated.

The sensitive nitro reductase thus appears to be a metalloflavoprotein with Mn as the possible metal constituent. Resistant reductase shows only a minimal requirement for cation, and this is taken to indicate a firm metal-protein-flavin complex. With the sensitive reductase, added  $FMNH_2$  can act as an electron source for nitro reduction, with the resistant reductase the flavoprotein is reduced by reduced diphosphopyridine nucleotide, and then reoxidized during nitro group reduction. The sulfhydryl requirements of the systems were investigated. The evidence indicates that the reductases are  $-SH$  enzymes.

The relationship of these findings to the mode of action of Aureomycin is discussed.

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# THE METABOLISM OF GLYCERIDE-GLYCEROL\*

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Various aspects of glycerol metabolism have been extensively investigated, both *in vivo* and *in vitro*, with the aid of isotopic glycerol. Experiments *in vivo* on the incorporation of  $C^{14}$ -glycerol into glycogen and various body lipides, as well as its oxidation to  $C^{14}O_2$  have been reported (1-3). The metabolism of  $C^{14}$ -glycerol by liver *in vitro* (4) and glycogen synthesis from deuterium-labeled glycerol (5) have also been studied. Recently there have been publications on some aspects of the metabolism of asymmetrically labeled glycerol (6-8). Further, glycerides synthesized from isotopically labeled glycerol have been utilized by many investigators as a tool in studying the mechanism of intestinal fat absorption (9-13).

The purpose of the present investigation was to obtain information on the metabolism of the glycerol moiety of triglycerides, or "glyceride-glycerol." Such a study should be germane since most of the glycerol that is normally ingested is esterified with fatty acids. Thus, data are presented on the oxidation of glyceride-glycerol and its incorporation into various lipid fractions of the gastrointestinal tract. In the light of the findings reported, the absorption and lipolysis of triglycerides are discussed. In addition, there are data on the incorporation of glyceride-glycerol carbon into plasma and liver lipides and into liver glycogen.

## EXPERIMENTAL

Normal male rats of the Wistar or Sprague-Dawley strains were used. The preliminary treatment of the animals was as described earlier (1), their weights ranged from 140 to 185 gm. These animals received by stomach tube glycerol-labeled triolein or glycerol-labeled tributyrin and were immediately placed in a metabolism cage. At the end of a specified time the rats were injected intraperitoneally with 20 mg of Amytal or Nembutal. When they reached surgical anesthesia, their abdominal and thoracic cavities were opened, and they were exsanguinated either by a heart puncture or by cutting the jugular veins. The liver, stomach, and

\* This work was supported in part by the United States Atomic Energy Commission, and by the Eugene Higgins Trust through Harvard University.

small and large intestines were removed. The percentage absorption of the glycerides, the oxidation of the glycerol moiety to  $\text{CO}_2$ , and its incorporation into liver glycogen were determined.

In a further series of experiments, 5 to 45 mg of  $\alpha\text{-C}^{14}$ -glycerol dissolved in 1 ml of water were administered by intragastric intubation to 24-hour fasted Sprague-Dawley male rats. These animals were placed in a metabolism cage, and their respiratory  $\text{C}^{14}\text{O}_2$  was collected for 4 hours.

### Methods

*Synthesis of Radioactive Triglycerides*—The tributyrin was prepared by reacting  $\alpha\text{-C}^{14}$ -glycerol (14) with butyryl chloride (15). Triolein was synthesized either by the method of Wheeler *et al* (16) using  $\text{C}^{14}$ -glycerol and oleic acid, or by treating the labeled glycerol with oleyl chloride<sup>1</sup> in a manner similar to the preparation of the tributyrin (15). Constants for the synthetic glycerides, such as saponification value (17), iodine value (18), and carbon content (19–21) were determined and found satisfactory.

*$\text{CO}_2$  and Glycogen Analyses*—The respiratory  $\text{CO}_2$  samples and the glycogen obtained were determined as previously described (1).

### Isolation of Lipides

*Gastrointestinal Contents*—The contents of the stomach were extracted with ether, and this extract was washed with water.

The small intestine was flushed with water until the washings were clear and colorless. Usually about 40 to 50 ml were required. The final pH was about 6.5. The aqueous solution was then extracted continuously with ether for 36 hours. After being washed with water, the ether was removed by evaporation, and the residue was extracted with chloroform. The large intestine and its contents were extracted with hot alcohol, followed by two extractions with alcohol-ether (2:1). This alcohol-ether solution was evaporated to dryness, and the residue was extracted with  $\text{CHCl}_3$ .

*Lipides from Tissues*—Lipides from the liver, washed stomach wall, and small intestine were obtained by mincing the tissue in a micro-Waring blender with a mixture of  $\text{CHCl}_3\text{-CH}_3\text{OH}$  (2:1), and the extracts obtained were filtered and washed according to Folch *et al* (22). The stomach lipides in early experiments and those from plasma were isolated as described in a previous publication (1).

The solutions of lipides in all instances were evaporated to dryness under  $\text{N}_2$  at about  $40^\circ$ . The residues were dried to constant weight, dissolved in  $\text{CHCl}_3$ , filtered, and made up to volume. Aliquots were then taken for

<sup>1</sup> The authors wish to thank Mr. William Spallina of Jacques Wolf and Company, Clifton, New Jersey, for the oleyl chloride.

phosphorus analyses, carbon determinations, or, in the case of the early experiments, direct plating for measurement of radioactivity

The phosphatide content of the lipides was estimated by total phosphorus analyses (23, 24) The lipides were fractionated into acetone-soluble and acetone-insoluble components as described previously (1) except that the acetone and  $MgCl_2$  were added to a chloroform solution rather than to a petroleum ether solution of the lipide A total of three fractionations was carried out to insure complete separation of the phosphatides from the neutral fats

*Glycerol Analyses*—In some experiments the ether-extracted aqueous washings from the small intestine were filtered and made up to volume This solution, or an aliquot thereof, was adjusted to pH 7 to 8, treated with periodic acid (25), and the resulting formaldehyde was precipitated by the addition of dimedon (1) After recrystallization and weighing, the derivative was converted to  $CO_2$  for determination of radioactivity In one experiment, carrier glycerol was added to an aliquot of the aqueous solution, and pure glycerol tribenzoate was prepared (2) The glycerol tribenzoate was saponified, and the liberated glycerol was periodate-oxidized The formaldehyde representing the  $\alpha$ -carbons of glycerol was collected as above

### *Measurement of Radioactivity*

All the samples from the early experiments with triglycerides were counted as described in previous papers (1, 26) The expired  $C^{14}O_2$  in later experiments and in the experiments with free glycerol was transferred to gas-counting tubes after manometric analysis (21) The samples of lipides and formaldimedon were converted to  $CO_2$ , and the  $C^{14}O_2$  counted in gas-counting tubes according to the method of Van Slyke *et al* (21)

### *Results*

*Absorption of Tributyrin and Triolein*—The radioactive lipides of the stomach and its contents, of the washings of the small intestine, and of the feces and other contents of the large intestine were considered unabsorbed The sum of these fractions subtracted from the total administered radioactivity was then considered to be the absorbed radioactivity Data on the intestinal absorption of tributyrin and triolein by different rats, determined as described above, are shown in Table I The relatively low absorption exhibited by the one rat studied at the 8 hour interval was anomalous and due to marked elimination of triolein in the feces

*Distribution of Unabsorbed Lipides*—After 1 hour, about 12 per cent of the administered activity remained in the stomach and about 15 per cent in the lumen of the small intestine There was but little change in these



TABLE I  
Intestinal Absorption of Tributyrin and Triolein

Fat	Duration of experiment	No of experiments	Fat given (mg per 100 sq cm B S A)*	Fat absorbed†	Index of absorption‡
	<i>hrs</i>			<i>per cent</i>	
Tributyrin	4	1	97	96	36.7
	6	1	43	98	40.0
Triolein	1	2	94 ± 3	73 ± 2	28.3 ± 0.6
	2	2	94 ± 1	75 ± 0	
	4	6	78 ± 6	95 ± 4	34.6 ± 2.2
	8	1	88	83	30.8

\* The means and average deviations from the means are given. The body surface area (B S A) was calculated by the method of Lee (27). The average body surface area in these experiments was  $268 \pm 12$  sq cm.

† The average amount of triolein administered was  $225 \pm 19$  mg.

‡ Defined as "mg of fat absorbed per 100 sq cm of body surface, for 100 mg of fat given."

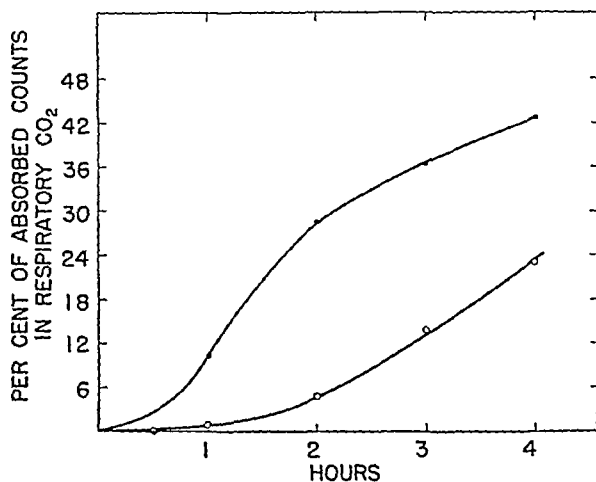


Fig. 1. Cumulative  $C^{14}O_2$  excretion after intragastric intubation of 22.5 mg of glycerol (in 1 ml of water), ●, and triolein, ○, to Sprague-Dawley rats. The points on the triolein curve are means of several animals. The average amount of triolein given was 240.7 mg, which corresponds to approximately 25 mg of free glycerol. In the experiment with free glycerol all the administered counts were considered to be absorbed.

distributions during the next hour. After 4 hours, however, only 2 per cent of the original activity appeared in each of the above fractions and in the feces.

*Oxidation of Glycerol Moiety of Triolein*—In Fig. 1 are shown cumulative  $C^{14}O_2$  excretion curves obtained after intragastric intubation of triolein or

an amount of glycerol approximately equivalent to the quantity of glycerol esterified in the administered triolein. Free glycerol was oxidized more rapidly than glyceride-glycerol.

*Lipolysis of Triolein*—It may be assumed that lipolysis of triolein precedes oxidation of the glycerol moiety. If this assumption is correct, then measurement of the oxidation of glycerol in an animal given a glycerol-labeled triglyceride compared to the oxidation of an equivalent amount of free  $C^{14}$ -glycerol would afford an *estimation* of the extent of complete<sup>2</sup> lipolysis, *i.e.* of free glycerol liberated from the triglyceride during the period of observation.

Thus, a family of curves was obtained for experiments of 1, 2, and 4 hours duration, in which the amount of free glycerol oxidized per 100 sq. cm. of body surface was plotted as a function of the quantity of free glycerol given. Such data had previously been obtained with rats of the Wistar strain (1), those for Sprague-Dawley rats are very similar. In experiments with triolein, the amount of fat which was absorbed and its equivalent in terms of glycerol were known. From measurements of the expired radioactive  $C^{14}O_2$  after administration of glycerol-labeled triolein, the amount of glycerol (a) that was oxidized could be ascertained. From the curve for free glycerol, it was possible to estimate how much oxidation would have occurred had *all* of the glycerol of the triglyceride been liberated, *i.e.* had the same amount of free glycerol been administered as was contained in the glyceride (b). By using these data a value  $((a)/(b) \times 100)$  could be obtained which is referred to as the "index of lipolysis."

Such calculations indicated that in experiments with triolein, the "index of lipolysis" varied from 9.0 to 15.7 per cent after 1 hour, from 11.2 to 16.5 per cent after 2 hours, and from 49.5 to 53.8 per cent after 4 hours. At 8 hours it was estimated to be 97 per cent.

*Free Glycerol in Lumen*—That complete intraluminary lipolysis to free glycerol and fatty acids might have occurred is demonstrated by the fact that there were found in the contents of the lumen small amounts of non-lipide, water-soluble formaldehydogenic substances which were radioactive. Addition of carrier glycerol to this water-soluble fraction, isolation of pure glycerol tribenzoate and determination of its activity showed that the greater part of the radioactive water-soluble glycol fraction was glycerol.

*Activity of Lipides of Intestinal Lumen*—In Table II are presented data on the activities of both acetone-soluble and acetone-insoluble lipides of the lumen of the small intestine. The relative specific activities of the glycerol of the neutral glycerides and of the phosphatides are compared.

<sup>2</sup> "Complete hydrolysis" is taken to mean hydrolysis of triglyceride to glycerol and fatty acids and "partial hydrolysis" as splitting to mono- and diglycerides, and fatty acids.

TABLE II  
*Relative Specific Activities of Lipides of Lumen of Small Intestine*

Duration of experiments	Weight of lipid	Per cent phosphatide	Relative specific activity of				
			Lipide	Neutral fat	Phosphatide	Neutral fat glycerol	Phosphatide glycerol <sup>a</sup>
hrs	mg		m $\mu$ c per mg carbon	m $\mu$ c per mg carbon <sup>†</sup>	m $\mu$ c per mg carbon <sup>†</sup>	m $\mu$ c per mmole <sup>‡</sup>	m $\mu$ c per mmole <sup>‡</sup>
1	58.6	19.3	0.54				
				0.58	1.03		0.79
1	67.9	13.8	0.65				
2	42.4	13.6	0.64				
				0.62	1.02	1.00	0.79
2	51.4	20.0	0.68				
4	10.2		0.32				
4	17.0	13.9	0.32				
8	4.7		0.01				

\* Calculated on the basis of 44 carbon atoms per molecule of phosphatide. All the activity was taken as being in the glycerol moiety.

† The specific activity of the administered triolein was taken as 1.00 m $\mu$ c per mg of carbon. Its actual value was 2.51 m $\mu$ c per mg.

‡ The specific activity of the glycerol from the administered triolein was taken as 1.00 m $\mu$ c per mmole. Its actual value was from 1630 to 1719 m $\mu$ c per mmole in different experiments.

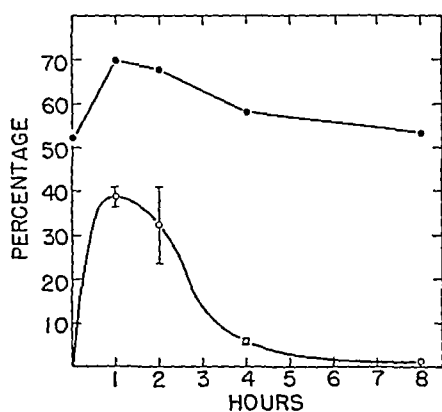


FIG 2

FIG 2 The disappearance of neutral fat from the small intestinal wall during absorption. The closed circles, ●, are the percentages of neutral fat in the intestinal lipides of these animals calculated by measuring the phosphatide content (23, 24) and subtracting this percentage from 100. The open circles, ○, represent the percentage of absorbed radioactivity in the intestinal neutral fat. The vertical lines are the ranges of two animals for each time period except in the case of the 8 hour experiment (one rat).

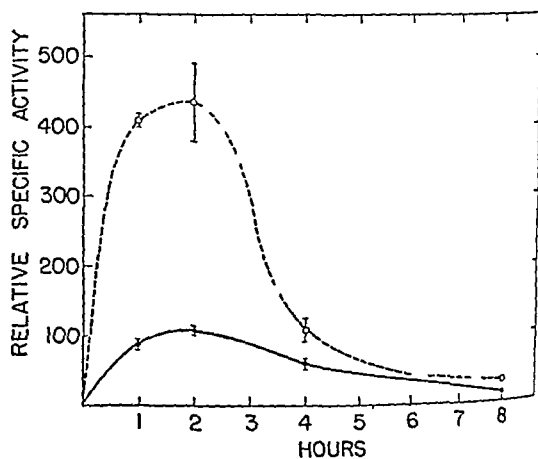


FIG 3

FIG 3 The specific activities of the acetone-soluble fats, ○, and phosphatides, ●, of the intestinal wall. Specific activity refers to the millimicrocuries per mg of carbon as a fraction of the specific activity of the administered triolein taken as 1.00. The vertical lines are the ranges of two animals for each time period except in the case of the 8 hour experiment (one rat).

*Radioactive Neutral Fats of Intestinal Wall*—The top curve in Fig 2 shows the increase and decrease in the neutral fat content of the intestinal wall lipides during absorption. The data at zero time were obtained from three rats which were sacrificed at the end of the fasting period. In Fig 2 are also plotted the percentages of the absorbed radioactivity in the neutral fat of the intestinal wall. A peak was reached at 1 hour, with the 2 hour value only slightly lower. However, the greatest change was observed

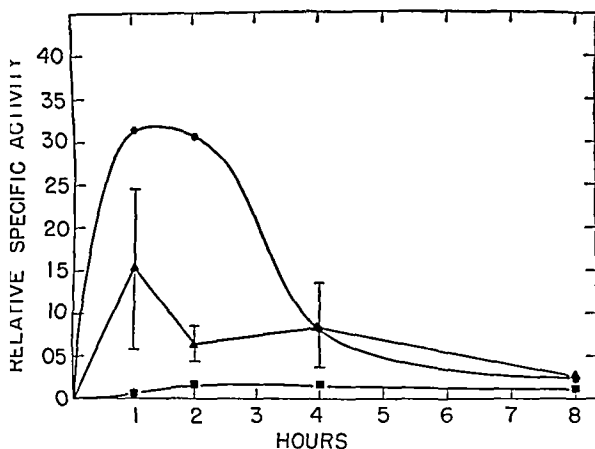


FIG 4

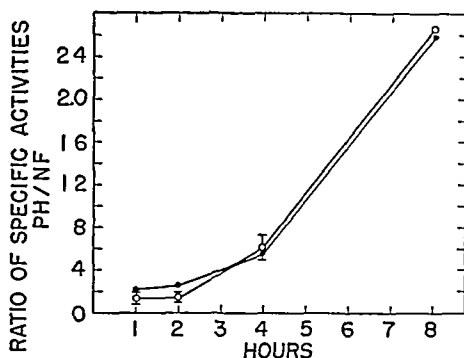


FIG 5

FIG 4 The specific activities of lipides of the wall of the small intestine, ●, plasma lipides, ▲, and liver lipides, ■. Specific activity refers to millimicrocuries per mg of carbon as a fraction of the specific activity of the administered triolein taken as 1.00. Two animals were used in each time period except in the case of the 8 hour experiment (one rat). The vertical lines are the ranges in those instances when the individual values were further than 0.03 mμc per mg of carbon apart. This occurred only in the plasma lipides.

FIG 5 Ratios of phosphatide (PH) specific activities to neutral fat (NF) (acetone soluble fat) specific activities for intestine, ●, and liver, ○. The vertical lines are the ranges of two liver lipid ratios for 1, 2, and 4 hours. The corresponding values for the intestinal lipides were in better agreement. Only one 8 hour experiment was carried out.

from 2 to 4 hours at the end of which time the neutral fat of the intestinal wall contained only about 2 per cent of the absorbed activity.

Fig 3 represents the changes with time in relative specific activity of the neutral fat and phosphatide fractions of the lipide of the intestinal wall after administration of glycerol-labeled triolein. During the first 3 hours the acetone-soluble fats were about 4 times as radioactive as the phosphatides, per mg of carbon. Although the amount of  $C^{14}$  in the phosphatides was significant, the total activity contributed by this fraction to the total intestinal wall lipid counts never amounted to more than a few per cent.

*Liver and Plasma Lipides*—Previous studies on the metabolism of free glycerol revealed that the liver lipides had a greater specific activity than

lipides from any other organ 6 hours after the administration of the  $C^{14}$  glycerol (1). In the present experiments it was observed that the liver lipides had extremely low specific activities. In Fig. 4 are plotted specific activity time curves for lipides extracted from the intestinal wall, plasma, and liver. The lipides from the intestinal wall had the greatest activity, with plasma lipide activities, which were somewhat variable, falling approximately between those of intestine and liver for most of the time. With regard to total activity, the percentage of absorbed counts in the plasma lipides was less than 0.5 in most instances, while the liver lipides contained about 0.6 to 2.0 per cent.

*Relationship of Neutral Fat and Phosphatide Activities*—A plot of the ratio of phosphatide activity to neutral fat activity at different times for both liver and intestine was made, and the results are shown in Fig. 5.

*Liver Glycogen*—Glycogen from the livers of several rats was isolated and counted. The amounts of glycogen found, the percentages of glycogen carbon derived from glyceride-glycerol carbon, and the amount of the glycerol converted to glycogen were extremely low as might have been expected in view of the small amount of liberated glycerol available to the organism for synthetic reactions. However, there was some incorporation, 0.1 to 0.5 per cent of glyceride-glycerol carbon being converted to glycogen carbon.

## DISCUSSION

*Extent of Absorption of Tributyrin and Triolein*—In the present experiments the extent of absorption of fat has been obtained from radioactivity data. A distinction should be made between the *radioactivity* absorbed and the *amount* of lipide absorbed. Lipolysis in the lumen, if it occurred, might liberate fatty acids, glycerol, and mono- and diglycerides which could be absorbed at different rates. Thus, if any freed glycerol were further metabolized in the lumen, or if it were absorbed more rapidly than a released fatty acid, then the percentage absorption of fat calculated from radioactivity data would be somewhat greater than the true absorption.

In expressing the extent of absorption of administered fat in this study the results were computed to 100 sq. cm. of body surface. Deuel *et al.* have demonstrated that data on the percentage of fat absorbed showed a greater uniformity when calculated in this way than when calculated on an absolute basis or on the basis of body weight (28). In the present work, where different amounts of lipide were administered to the individual animals, it was found advantageous to compute the data also on the basis of the amount of fat given. Thus, an "index of absorption" has been found useful and is defined as "mg. of fat absorbed per 100 sq. cm. of body surface, for 100 mg. of fat administered" (See Table I).

The results for the absorption of tributyrin are in accord with previously

reported data (29, 30) The magnitude of the "index of absorption" for triolein reported in this work may be compared with that calculated from data of Deuel *et al* (28) and Steenbock *et al* (31) for natural fats In general, the differences found may be explained by the fact that the workers mentioned administered from 4 to 6 times as much lipide as was given in the present experiments On the other hand the data for the percentage absorption of triolein in 4 hours (Table I) are in agreement with those of Borgstrom on the absorption of corn oil administered at a level of 0.1 ml per 100 sq cm, which more closely approximates the conditions employed by the present authors (32)

On the basis of the values in Table I it would appear that during the 2nd hour after administration of lipide the passage of fat from the lumen into the intestinal mucosa was slowed down, or was at a standstill This may be attributed to a mechanism which would permit the absorption of fat from the lumen only when the already absorbed fat in the mucosa was further metabolized or removed by the transporting system (lacteals)

*Lipolysis of Administered Triglyceride*—From the values for the "index of lipolysis" given, there appeared to be a considerable lipolysis of the ingested triolein in the 1st hour This hydrolysis was probably partly intraluminary and served to furnish emulsifying agents, *i e* fatty acids and monoglycerides (33) During the 2nd hour the lipolysis appeared to be slowed down considerably, as was absorption (see above) From the 2nd to the 4th hour there was again a very notable degree of lipolysis, the "index of lipolysis" amounting to a total of about 50 per cent after 4 hours

The fact that there was about a 75 per cent absorption and a calculated 12 per cent lipolysis of absorbed triolein at 1 and 2 hours indicates that a relatively large amount of fat passed from the lumen into the mucosal cells in an unhydrolyzed or partially hydrolyzed, *i e* mono- or diglyceride form, a fact which is now generally accepted (33, 34) In the case of the tributyrin experiments, the "indices of lipolysis" appeared to be 100 per cent and 86 per cent in the two experiments reported (Table I), which is consistent with data obtained by others with short chain triglycerides

Several factors should be pointed out regarding the interpretation of the "index of lipolysis" First, factors affecting the rate of liberation and oxidation of the *freed* glycerol make the values only approximate Secondly, it is assumed that the glycerol must be freed before it is oxidized Thirdly, the calculations are based on *absorbed* triolein However, any triolein completely lipolyzed in the *lumen* would have been unabsorbed triolein and the glycerol liberated might then have been rapidly absorbed and oxidized Under such circumstances it would be more accurate to calculate the "index of lipolysis" on the basis of administered triolein, and one would obtain values lower than those quoted

The most active periods of lipolysis would appear to have been at those

times when the glycerides were in the process of leaving the mucosal cells, as shown by the decrease both in neutral fat and radioactivity (Fig 2). This suggests that the major part of the lipolysis up to 4 hours took place in the mucosal cells. Resynthesis of glycerides from released fatty acids and endogenous glycerol (non-labeled) must also have taken place since triglycerides are the predominant acetone-soluble lipides in lymph (3a). All these data agree in essence with the findings of Borgstrom, who demonstrated that the amount of lymph neutral fat reached a maximum during the 2nd and 3rd hour of absorption (32), and also with the suggestion of Reiser that "an intracellular mechanism regulates the hydrolysis" (of glycerides) (36). Conceivably the cramming of unsplit or partially split triolein into the mucosal cells slowed down hydrolysis until some of the fat passed into the lymph or had undergone further reactions, *e g* partial hydrolysis. On the other hand, as suggested by Borgstrom (37), the orientation of absorbed monoglycerides (monoolein) with their hydroxyl groups against the water phase (in the cell) could protect the other glycerides from the action of the enzymes. Other factors such as the nature of the fat or the composition of the mixture of fats containing the labeled material are undoubtedly also of importance (38).

The fact that after 4 hours there was relatively little radioactive lipid in the intestinal wall would lead one to suspect that much of the hydrolysis of the triolein after 4 hours occurred in extraintestinal locales.

*Activity of Acetone-Soluble Lipides of Intestinal Lumen*—The data in Table II shed further light on intraluminary lipolysis. Acetone soluble lipides of the lumen exhibited a relatively low specific activity because of dilution with what probably were fatty acids released during lipolysis. This was confirmed by the fact that the specific activity of the isolated glycerol moiety of the lumen neutral fat at 2 hours was identical to that of the glycerol moiety of the ingested triolein (seventh column, Table II). Thus, recombination of released fatty acids with endogenous glycerol or glycerol precursors to form neutral fat did not take place in the lumen, nor was there dilution with endogenous glycerides. This is in line with Borgstrom's observation that free glycerol does not participate in intraluminary esterification (11). The free fatty acids which dilute the glyceride activity could have originated from the splitting of triolein, followed by absorption of the split products at unequal rates. That the activities of the fractions did not change appreciably from 1 to 2 hours (fifth column, Table II) supports the evidence provided by the data on absorption and the "index of lipolysis" that there was virtually a plateau in the 2nd hour with respect to the ability of the animal to metabolize administered triolein.

*Phosphatide Synthesis in Intestinal Wall and Lumen*—It is generally believed that phosphorylation is not an essential step in the transport of

fat across the intestinal wall (39, 40), and it has been shown that only a few per cent of absorbed fatty acids are found in lymph phosphatides (41, 42) Reiser and Dieckert have recently reported an observation made after administering  $C^{14}$ -glycerol-labeled fats In their experiments there was less activity in mucosal and lymph phosphatide glycerol than in that of triglycerides, and these workers suggested that "resynthesized triglycerides must be the precursors of mucosa and lymph phospholipides, rather than the converse" (43) The curves of Fig 3 are consistent with these observations

Recently Buensod *et al* administered free deuterium-labeled glycerol or deuterium-labeled glycerophosphate to rats together with free fatty acids or fat (12) From the isotopic measurements 3 hours later it was concluded that neither glycerol nor glycerophosphate were important precursors of intestinal phosphatide In these experiments the phosphatides never contained more than about 0.1 per cent of the deuterium of the original labeled glycerol A very low incorporation of free glycerol into intestinal lipides was also observed earlier by the present investigators (1) On the other hand the relative specific activity of the phosphatides of the intestinal wall at 3 hours in Fig 3 was about 0.084  $m\mu c$  per mg of carbon or, in other words, 8.4 per cent of the glycerol of these intestinal phosphatides was derived from the ingested triolein If, as suggested by the above workers, neither glycerol nor glycerophosphate are precursors of phosphatide in the intestine, then there arises the question of how the phosphatides in the present experiments were formed

It is possible that triglycerides are precursors of these phosphatides, as suggested by Reiser and Dieckert (43) or, in addition to intracellular synthesis of phosphatides, phosphatides could have been formed in the lumen, and absorbed with glycerides Evidence that there might have been intraluminary phosphatide synthesis is derived from Table II

The results in the fifth through the seventh columns of Table II show that the phosphatides, as well as the neutral fats of intestinal lumen contents, were labeled The relatively high specific activity of the phosphatides suggests that they could have been formed directly from the administered triolein by interesterification with phosphate or a phosphorylated compound A reaction less likely in such a locale as the lumen was suggested by Chaikoff who postulated that a high energy phosphate compound could react with a triglyceride molecule in a transfer type of reaction to produce a phosphatidic acid (44)

A further point of considerable interest concerning intestinal wall and liver phosphatides arises from the data of Fig 5 Although the activities of the total lipides of the intestinal wall and the liver differed widely (Fig 4), the ratios of activity of the phosphatides to the activities of the neu-



tial fats in the two organs were very similar at any given time. This could mean that there exist in both the intestinal wall and the liver similar mechanisms for the transformation of glycerides to phosphatides. Moreover, the slopes of the curves of Fig 5 and the shapes of those of Fig 3 indicate that neutral fat turnover is more rapid than phosphatide turnover in these organs.

In general, the data reported support the main feature of the views put forward by Frazer and now commonly accepted, *i.e.* total lipolysis is not a prerequisite of absorption (45, 46). Details on events in the intestinal lumen and wall during absorption have been provided. For example, some complete lipolysis does apparently occur in the lumen, contrary to *in vitro* evidence of Frazer and Sammons (47), but in conformity with experiments of Borgstrom (48). Further, lipase-catalyzed reactions such as those reported by Borgstrom *in vivo* and *in vitro* (48) with respect to the incorporation of labeled free fatty acids into glycerides, have been suggested as causing the incorporation of neutral glyceride-glycerol into phosphatide-glycerol in the intestinal lumen *in vivo*. Finally, a temporal differentiation of three phases of absorption, an initial rapid phase, a second sluggish phase, and a renewal of the more rapid phase, poses a number of questions concerning the absorption of fat administered alone and the metabolism of glyceride-glycerol. It would be important to make comparisons with situations in which fat is administered in conjunction with protein and carbohydrate.

#### SUMMARY

- 1 The metabolism of glyceride-glycerol has been studied after oral administration of tributyrin or triolein labeled with  $C^{14}$  in the glycerol moiety.

- 2 After 1 or 2 hours the absorption of triolein was approximately the same, *i.e.*  $74 \pm 2$  per cent had been absorbed, while  $95 \pm 4$  per cent was absorbed by the end of 4 hours. Absorption of tributyrin was nearly complete at this time.

- 3 At the end of 4 hours approximately one-quarter of the absorbed glyceride-glycerol of triolein had been oxidized.

- 4 During the 1st hour lipolysis appeared to be considerable, amounting to about 12 per cent of lipide absorbed. After 2 and 4 hours the "index of lipolysis" had risen to approximately 14 and 52 per cent, respectively.

- 5 During the 2nd hour after lipide administration a plateau was reached with respect to absorption, lipolysis, and disappearance of labeled lipide from the intestinal wall. In the 3rd and 4th hours these activities were greatly increased.

- 6 Free  $C^{14}$ -glycerol was isolated from the contents of the lumen of a rat sacrificed 2 hours after administering glycerol-labeled triolein. Also found

in the lumen of rats killed after 1 and 2 hours were phosphatides whose relatively high specific activity suggested that they were formed from glycerides

7 There was little synthesis of liver glycogen from glyceride-glycerol

It is a pleasure to express our gratitude to Dr Donald D Van Slyke and Professor A Baird Hastings for their helpful advice, and to Mr John A Plazin for his assistance in carrying out many of the carbon combustions during the course of this investigation

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boxymuconic acid and  $\beta$ -carboxymuconolactone, however, no effect was found with the addition of sulphhydryl inhibitors. Thus the observed inhibition was on the initial step in the reaction sequence.

*Role of Ferrous Ion in Protocatechuic Acid Oxidase*—A stimulating effect of  $\text{Fe}^{++}$  was noted during the purification of the enzyme. Certain preparations which had been fractionated failed to show any increase in specific activity. Addition of  $\text{Fe}^{++}$  to these preparations caused a marked stimu-

TABLE I  
*Inhibition and Reactivation of Protocatechuic Acid Oxidase  
by Sulphydryl Reagents*

$\text{HgCl}_2$  and *p*-chloromercuribenzoate were added 5 minutes before the substrate in the spectrophotometric assay and 10 minutes before the substrate in the manometric assay. In the experiments in reactivation, reduced glutathione was added 5 minutes after the inhibitors. Both assays were run in the presence of tris(hydroxymethyl)aminomethane buffer (0.1 M, pH 7.0). The enzyme preparation was the 0.3 to 0.7  $(\text{NH}_4)_2\text{SO}_4$  fraction.

Substance tested	Final concentration	Assay	Control activity
	M		per cent
$\text{HgCl}_2$	$2 \times 10^{-4}$	Spectrophotometric	60
"	$2 \times 10^{-4}$	"	100
" + reduced glutathione	$1 \times 10^{-3}$		
<i>p</i> -Chloromercuribenzoate	$2 \times 10^{-4}$	"	34
"	$2 \times 10^{-4}$	"	73
" + reduced glutathione	$1 \times 10^{-3}$		
Reduced glutathione	$1 \times 10^{-3}$	"	100
<i>p</i> -Chloromercuribenzoate	$1.7 \times 10^{-4}$	Manometric	32
"	$1.7 \times 10^{-4}$	"	102
" + reduced glutathione	$3 \times 10^{-3}$		

lation (Table II), and its addition to crude extracts resulted in a similar stimulation. The effect of  $\text{Fe}^{++}$  was specific (Table III), although the addition of  $\text{Fe}^{+++}$  and  $\text{Co}^{++}$  stimulated the preparations somewhat. The amount of  $\text{Fe}^{++}$  required for stimulation was small. Addition of  $1 \times 10^{-3}$  M  $\text{Fe}^{++}$  resulted in a 24 per cent increase in activity. Additions of *o*-phenanthroline and  $\alpha, \alpha$ -dipyridyl, both at concentrations of  $1 \times 10^{-3}$  M, resulted, respectively, in 73 and 68 per cent inhibition of protocatechuic acid oxidase in the absence of added  $\text{Fe}^{++}$ . Cyanide at similar or higher concentrations was without effect.

The addition of  $\text{Fe}^{++}$  to preparations which degraded  $\beta$ -carboxymuconic acid and  $\beta$ -carboxymuconolactone had no stimulatory effect on the rate of

treated with solid  $(\text{NH}_4)_2\text{SO}_4$ . The material which precipitated between 0.3 and 0.7 saturation was collected by centrifugation and dissolved in water (10 ml per gm of dried mycelia). The ammonium sulfate precipitate was further purified by dialysis against water, followed by  $\text{Ca}_3(\text{PO}_4)_2$  gel absorption. The enzyme was eluted from the gel with potassium phosphate buffer (0.1 M, pH 7.0) and again dialyzed against distilled water. All operations were carried out at 5°.

*Analytical*—The oxidation of protocatechuic acid was followed by two methods, oxygen uptake at 33° and spectrophotometric assay at room temperature.

*Manometric Assay*—The reactions were carried on by using conventional Warburg techniques at 33°, with air as the gas phase. The substrate concentration was  $2.67 \times 10^{-3}$  M.

*Spectrophotometric Assay*—Enzyme preparations were assayed in a Beckman DU spectrophotometer in 0.5 cm silica cells by the change in optical densities at 290, 270, and 220 m $\mu$  at room temperature. The molecular extinction coefficients used were those previously described (1, 4). The substrate concentration was  $1.7 \times 10^{-4}$  M.

A unit of specific activity is defined as the oxidation of 0.1  $\mu$ mole of protocatechuic acid per hour per mg of protein. The ammonium sulfate fraction had 6.5 units and the  $\text{Ca}_3(\text{PO}_4)_2$  gel eluate had 10.5 units as measured by the manometric assay. The spectrophotometric assay gave lower and variable values for specific activity because of the difference in temperature.

Protein was determined by the method of Gornall, Bardawill, and David (5).

## RESULTS AND DISCUSSION

*Requirement for Sulfhydryl Groups*—Inhibition of the enzyme preparation by  $\text{Hg}^{++}$  and *p*-chloromercuribenzoate and the reversibility of the inhibition by reduced glutathione (Table I) strongly suggest the presence of essential thiol groups on the enzyme. The failure to observe inhibition with iodoacetate and *N*-phenyl maleimide (1) does not seem unusual for this type of enzyme. Crandall (6) reports that crude preparations of homogentisic acid oxidase are not inhibited by iodoacetate but are sensitive to *p*-chloromercuribenzoate. However, the addition of reduced glutathione failed to stimulate any preparation of protocatechuic acid oxidase as it does in the case of homogentisic acid oxidase (6, 7).

Since our enzyme preparations contained the enzymes which attack  $\beta$ -carboxymuconic acid and  $\beta$ -carboxymuconolactone, it seemed possible that the effect of the sulfhydryl inhibitors upon protocatechuic acid oxidase might be indirect. Upon measuring the rate of disappearance of  $\beta$  car

booxymuconic acid and  $\beta$ -carboxymuconolactone, however, no effect was found with the addition of sulfhydryl inhibitors. Thus the observed inhibition was on the initial step in the reaction sequence.

*Role of Ferrous Ion in Protocatechuic Acid Oxidase*—A stimulating effect of  $\text{Fe}^{++}$  was noted during the purification of the enzyme. Certain preparations which had been fractionated failed to show any increase in specific activity. Addition of  $\text{Fe}^{++}$  to these preparations caused a marked stimu-

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The addition of  $\text{Fe}^{++}$  to preparations which degraded  $\beta$ -carboxymuconic acid and  $\beta$ -carboxymuconolactone had no stimulatory effect on the rate of

disappearance of these compounds. These results indicate that the effect of  $\text{Fe}^{++}$  was on the initial step only in the breakdown of protocatechuic acid.

TABLE II

*Stimulating Effect of Ferrous Ion on Protocatechuic Acid Oxidase*

Ferrous ion was added 30 minutes prior to substrate in the manometric assay, while in the spectrophotometric assay the preincubation period was 15 minutes. Both preincubations were carried out at  $5^\circ$ . All enzyme preparations gave essentially the same response. In the manometric assay tris(hydroxymethyl)aminomethane buffer (0.1 M, pH 7.0) was used, in the spectrophotometric assay no buffer was used. The results below were obtained with a dialyzed  $\text{Ca}_3(\text{PO}_4)_2$  gel eluate.

Addition	Final concentration	Assay	Control activity
	<i>M</i>		<i>per cent</i>
$\text{FeSO}_4$	$1 \times 10^{-3}$	Manometric	290
"	$1 \times 10^{-4}$	"	300
"	$1 \times 10^{-6}$	"	184
"	$1 \times 10^{-8}$	"	124
"	$1 \times 10^{-10}$	"	100
"	$1 \times 10^{-3}$	Spectrophotometric	270

TABLE III

*Effect of Metal Ions on Protocatechuic Acid Oxidase*

The various metal ions were added 30 minutes prior to the substrate. The preincubation was carried out at  $5^\circ$ . The enzyme preparation used was a dialyzed gel eluate. The assays were run in the presence of tris(hydroxymethyl)aminomethane buffer (0.1 M, pH 7.0). The manometric assay was used.

Cation tested	Final concentration	Control activity
	<i>M</i>	<i>per cent</i>
$\text{Fe}^{++}$	$1 \times 10^{-4}$	252
$\text{Fe}^{+++}$	$1 \times 10^{-4}$	151
$\text{Ca}^{++}$	$1 \times 10^{-4}$	97
$\text{Al}^{+++}$	$1 \times 10^{-4}$	92
$\text{Mn}^{++}$	$1 \times 10^{-4}$	77
$\text{Mg}^{++}$	$1 \times 10^{-4}$	94
$\text{Co}^{++}$	$1 \times 10^{-4}$	123
$\text{Cu}^{++}$	$1 \times 10^{-4}$	92

In the absence of added  $\text{Fe}^{++}$ , an absorption peak at 408 to 410  $\text{m}\mu$  was observed in purified preparations. However, upon the addition of  $\text{Fe}^{++}$  ( $1 \times 10^{-3}$  M) this peak disappeared and two shoulders appeared at 385 to 400 and 470 to 485  $\text{m}\mu$ . The cause of this spectral shift is not known.

The addition of protocatechuic acid to the  $\text{Fe}^{++}$ -treated enzyme failed to change the spectrum

The results presented above indicate that protocatechuic acid oxidase from *Neurospora* has requirements for  $\text{Fe}^{++}$  and sulfhydryl groups similar to those described for enzymes which catalyze the enzymatic oxidative cleavage of certain phenols (6). The failure of cyanide to inhibit protocatechuic acid oxidase and the failure of reduced glutathione to activate protocatechuic acid oxidase in the absence of sulfhydryl inhibitors, even in the presence of  $\text{Fe}^{++}$ , are two major differences between protocatechuic acid oxidase and homogentisic acid oxidase.

#### SUMMARY

Protocatechuic acid oxidase isolated from *Neurospora* requires the addition of ferrous ion for maximal activity. The addition of  $1 \times 10^{-6}$  M ferrous ion resulted in an 84 per cent increase in activity.

The addition of *p*-chloromercuribenzoate and of mercuric ion resulted in a marked decrease in enzymatic activity.

These results indicate that ferrous ion and sulfhydryl groups form integral parts of the oxidizing enzyme which transforms protocatechuic acid to *cis,cis*- $\beta$ -carboxymuconic acid.

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# THE SPECTROPHOTOFUOROMETRIC DETERMINATION OF TRYPTOPHAN IN PLASMA AND OF TRYPTOPHAN AND TYROSINE IN PROTEIN HYDROLYSATES

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A spectrophotofluorometer capable of continuous activation of solutions and measurement of resultant fluorescence throughout the quartz-ultra-violet and visible regions of the spectrum has been previously described (1). The ability of such an instrument to deliver high intensity monochromatic light at the absorption maxima of tryptophan and tyrosine, and to measure the resulting ultraviolet fluorescence spectra, provides the basis for a simple and extremely sensitive method for the determination of these amino acids in tissues and in protein hydrolysates.

## EXPERIMENTAL

*Spectrophotofluorometer*<sup>1</sup>—The design and operation of this instrument are described in detail elsewhere (1). It consists essentially of a high intensity xenon arc source which emits continuously throughout the ultra-violet and visible regions and two monochromators, one to isolate monochromatic light for activation and the other at right angles to the first to analyze the emitted fluorescence, a photomultiplier tube<sup>2</sup> is used to measure the intensity of the fluorescent light. Both activation and fluorescence spectra may be rapidly displayed on the screen of a cathode ray oscilloscope or the chart of a pen and ink recorder. Activation and fluorescence spectra for tryptophan and tyrosine are given in Figs. 1 and 2. The intensity of fluorescence of both amino acids is proportional to concentration throughout the range of concentration employed in these studies.

### *Tryptophan and Tyrosine in Protein Hydrolysates*

A survey of all of the naturally occurring amino acids found in proteins revealed that only tyrosine and tryptophan exhibit detectable fluorescence in aqueous media. Fig. 3 shows the fluorescence spectra of pure samples

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<sup>1</sup> The experimental instrument (1) has been used interchangeably with commercial spectrophotofluorometers obtained from the American Instrument Company and the Farrand Optical Company, Inc.

<sup>2</sup> RCA 1P28 photomultiplier.

of these two amino acids and of mixtures of the two at various pH values. At pH 11, the intensity of the tryptophan fluorescence is approximately 100 times greater than that of a corresponding concentration of tyrosine, and the ability of a spectral instrument to resolve the two fluorescence bands whose maxima occur 50  $m\mu$  apart makes it possible to measure tryptophan in the presence of large excesses of tyrosine. Since tryptophan is destroyed by acid hydrolysis, the tyrosine content of proteins containing any amount of tryptophan may be readily determined without interference

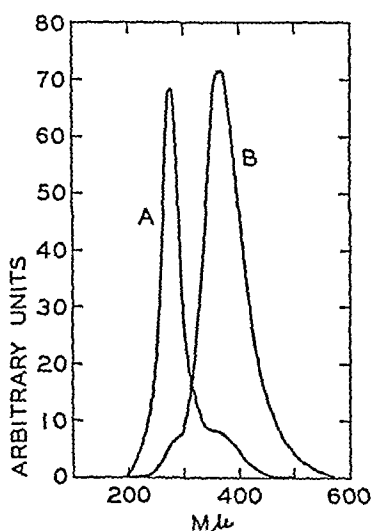


FIG 1

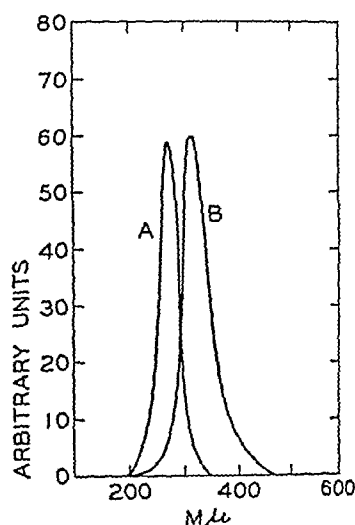


FIG 2

FIG 1 Activation and fluorescence spectra of tryptophan. Curve A, activation spectrum for pure tryptophan (0.1  $\gamma$  per ml) in 0.5 M  $\text{Na}_2\text{CO}_3$  obtained by setting fluorescence monochromator constant at 360  $m\mu$  and scanning with activating monochromator. Curve B, fluorescence spectrum for same sample with activation monochromator set at 280  $m\mu$  while scanning with fluorescence monochromator.

FIG 2 Activation and fluorescence spectra of tyrosine. Curve A, activation spectrum of pure tyrosine in phosphate buffer, pH 8.0, Curve B, fluorescence spectrum of same. The activation and fluorescence spectra were obtained as for tryptophan with monochromators set at 310 and 275  $m\mu$ , respectively.

**Materials**—Pure crystalline samples of reference proteins were employed, zinc insulin, lot No. 535664 was obtained from Eli Lilly and Company, bovine serum albumin, lot No. 370-295-B was obtained from Armour and Company, crystalline  $\beta$ -lactoglobulin was made available by Dr. Thomas L. McMeekin of the Eastern Utilization Research Branch, United States Department of Agriculture.

Tryptophan and tyrosine standards were prepared daily by dilution of stock solutions<sup>3</sup> of the L-amino acids obtained from the Nutritional Biochemicals Corporation.

<sup>3</sup> Stock solutions of 1 mg of amino acid per ml of 0.1 N  $\text{NH}_4$  were employed. These were kept refrigerated and were freshly prepared each week.

**Basic Hydrolysis**—The protein is dissolved in 10 ml of 5.0 N NaOH and heated in an open tube<sup>4</sup> in an autoclave for 20 hours at 2 atmospheres. The amount of protein taken (20 to 50 mg)<sup>5</sup> is corrected for moisture content as determined by drying a separate sample *in vacuo* at 100°. The cooled hydrolysate is acidified with 1.5 ml of 5 N H<sub>2</sub>SO<sub>4</sub>, transferred quantitatively to a 25 ml volumetric flask, and made up to the mark with water. The acidified solution is clarified by centrifugation. A tryptophan standard (1 to 2  $\gamma$ ) and a blank are carried through the hydrolysis procedure.<sup>6</sup>

**Acid Hydrolysis**—The protein sample is heated as above in 10 ml of 7 N H<sub>2</sub>SO<sub>4</sub>, neutralized by the dropwise addition of ammonium hydroxide, and diluted to 25 ml with water.

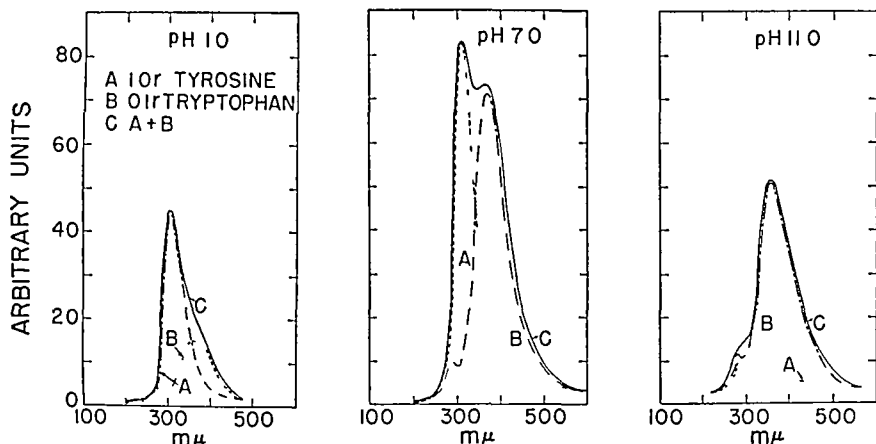


FIG 3 Variation of tryptophan and tyrosine fluorescence with pH. Activating wave length = 280 m $\mu$  in each case. pH 10 = 0.1 N H<sub>2</sub>SO<sub>4</sub>, pH 7.0 = phosphate buffer, pH 11.0 = 0.09 N NH<sub>3</sub>.

**Determination of Tryptophan**—An aliquot of the acidified alkaline hydrolysate (8.0 ml) is diluted to 50 ml with N sodium carbonate. The final solution (133 to 333  $\gamma$  of protein per ml) is activated at 280 m $\mu$  and its fluorescence at 360 m $\mu$  is compared to that of an appropriate standard.

**Determination of Tyrosine**—An aliquot of the neutralized acid hydrolysate is diluted 10-fold with phosphate buffer, pH 8.0, and activated at

<sup>4</sup> Test tubes of Corning brand No. 7280, alkali-resistant glass are used. The use of standard Pyrex tubes for alkaline hydrolysis results in a high degree of light scattering in the final solution owing to the presence of colloidal silicate. Scattering of the fluorescent light may lead to low results, as much as 20 per cent in error. High speed centrifugation (about 30,000 r.p.m.) can be used to minimize light scattering.

<sup>5</sup> Quantities of this magnitude were used in these studies because of the availability of the protein employed. The sensitivity of the method is such, however, that samples as small as 100  $\gamma$  may be carried through the same dilution process.

<sup>6</sup> The tryptophan standard is subjected to hydrolysis conditions to correct for losses (5 to 10 per cent) during alkaline hydrolysis.

275 m $\mu$ , and its fluorescence at 310 m $\mu$  is compared to that of a standard solution of tyrosine (1 to 2  $\gamma$  per ml)

*Specificity*—Full activation and fluorescence spectra of the diluted hydrolysates proved identical with those of authentic samples of the respective amino acids determined under the same conditions. The ease with which full spectra may be obtained makes possible their application to each

TABLE I

*Tyrosine and Tryptophan Contents of Known Amino Acid Mixtures*

The values are given in gm per 100 gm of mixture

Sample No	Tyrosine		Tryptophan	
	Added	Found	Added	Found
I	11.8	12.1	1.08	1.01
II	6.31	6.22	4.36	4.25
III	9.80	9.85	0.0	0.0
IV	0.0	0.0	2.92	2.75

TABLE II

*Tyrosine and Tryptophan Contents of Protein*

The values are given in gm per 100 gm of protein

Protein	Tyrosine		Tryptophan	
	Found	Given in literature	Found	Given in literature
Insulin	11.6	12.25 (2)*	0	0 (3)
Bovine serum albumin	6.0	5.5 (4)	0.71	0.6 (4)
$\beta$ -Lactoglobulin	3.75	3.69 (5)	1.75	1.92 (5)

\* Bibliographic reference

individual analysis. Thus the presence of extraneous fluorescent or absorbing materials may be readily detected from the shape of the curve.

*Results*—Known amounts of tyrosine and tryptophan when added to mixtures of amino acids<sup>7</sup> and subjected to hydrolytic conditions were quantitatively recovered (Table I). In addition, the tryptophan and tyrosine contents of crystalline samples of insulin, bovine serum albumin, and  $\beta$ -lactoglobulin were determined and found to be in good agreement with values cited by other workers (Table II).

<sup>7</sup> Synthetic mixtures of pure amino acids were prepared which contained approximately 5 per cent by weight of each of the following: glycine, alanine, valine, leucine, isoleucine, aspartic acid, glutamic acid, histidine, lysine, serine, threonine, proline, hydroxyproline, phenylalanine, methionine, cysteine, asparagine, and glutamine. Known amounts of tryptophan, tyrosine, or both were then added.

*Tryptophan in Plasma*

Plasma proteins are precipitated according to the procedure of Dunn *et al* (6). The supernatant solution is brought to pH 11 and activated at 280  $m\mu$ . The resultant single fluorescence band which specificity studies showed to arise solely from tryptophan is measured at 360  $m\mu$ .

*Method*—A 1.0 ml sample of plasma is diluted with 4 volumes of water and acidified with 0.5 ml of 0.6  $N$   $H_2SO_4$ . Protein is precipitated by the addition, with constant shaking, of 0.5 ml of 10 per cent sodium tungstate<sup>8</sup> and is removed by centrifugation. A 3.0 ml aliquot of the clear supernatant fluid is transferred to a small centrifuge tube containing 1.0 ml of 0.25  $M$  barium chloride and the precipitate of barium sulfate and barium tungstate is removed by centrifugation.<sup>9</sup> A 2.0 ml aliquot of the solution is treated with 0.5 ml of 2  $M$  sodium carbonate to precipitate excess barium ion and bring the pH of the sample to the optimal value for tryptophan fluorescence. After centrifugation for 10 minutes at 3000 r.p.m., a small volume (1 to 1.5 ml), which should be clear and colorless,<sup>10</sup> is carefully withdrawn and its fluorescence is measured as described for protein hydrolysates.

*Standards*—The above procedure involves an over-all dilution of 1:10. Since the normal plasma tryptophan level in fasting subjects is quite constant at about 10  $\gamma$  per ml, a standard solution of 1.0  $\gamma$  of pure tryptophan per ml in 0.3  $M$  sodium carbonate will, therefore, give a reading close to that of the sample. As an additional check, internal standards are routinely used. A sample in which pure water is substituted for plasma is carried through the entire procedure to serve as a fluorescence blank. A typical determination in which full fluorescence spectra of sample, internal and absolute standards, and blank are employed is illustrated in Fig. 4.

*Specificity*—That tryptophan is the only component normally present in these plasma filtrates in concentrations high enough to show a measurable fluorescence under the conditions employed was indicated by a number of experiments. A 1:10 protein-free filtrate and a 1.0  $\gamma$  per ml solution of pure tryptophan, each buffered at pH 4.0, were subjected to a nine plate countercurrent distribution, with *n*-butanol as the organic phase. The aqueous phases from each tube were made alkaline by the addition of 1 volume of 2  $N$  ammonium hydroxide, and the fluorescence was measured as described above. The distribution of the plasma fluorophor closely

<sup>8</sup> Reagent grade  $Na_2WO_4 \cdot 2H_2O$

<sup>9</sup> Tungstate ion has a slight quenching effect upon tryptophan fluorescence and must be removed.

<sup>10</sup> If the blood sample has suffered extensive hemolysis, varying amounts of pigments absorbing in the region of tryptophan fluorescence will carry through the procedure and result in low readings. Although the use of internal standards will correct for this source of error, such samples should be avoided whenever possible.

approximated that of pure tryptophan, with a maximal concentration of fluorescent material appearing in the fifth tube in each case

As is the case with many ampholytic fluorophors, the intensity of tryptophan fluorescence varies markedly with pH. The fluorescence and activation spectra of the filtrate were, therefore, compared to those of authentic tryptophan samples at various pH values. A sharp peak fluorescence was observed in each case at pH 11. Although several other indole derivatives have the same activation and fluorescence maxima as tryptophan, the

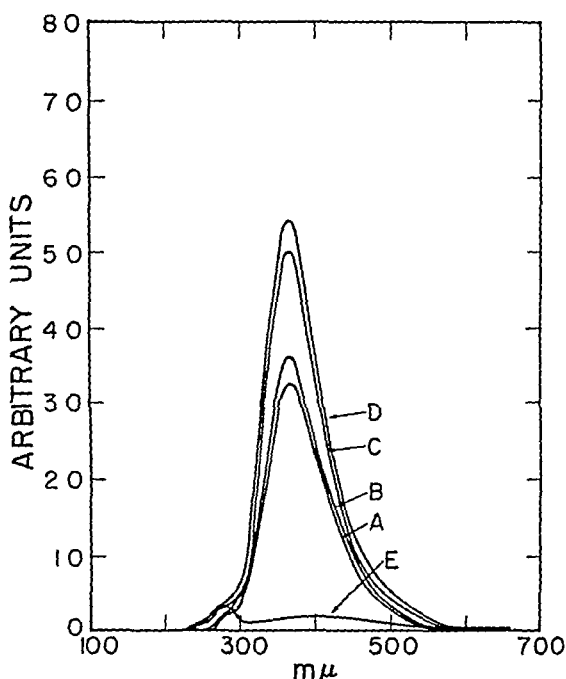


FIG 4 Curve A, absolute standard, 10  $\gamma$  per ml, Curve B, plasma, Curve C,  $\frac{1}{2}$  Curve A, Curve D, plasma +  $\frac{1}{2}$  Curve A, Curve E, blank

variations in fluorescence intensity with changing pH are markedly different for each compound. Finally, the fluorescence of both authentic tryptophan and the plasma fluorophor was quantitatively quenched by ascorbic acid, inorganic nitrate, thiosulfate, and peroxide.

### Results

Recovery of added amounts of tryptophan from plasma was found to be excellent. When 2.5 to 20  $\gamma$  of tryptophan were added to 1.0 ml aliquots of plasma, the mean recovery was 98.7 per cent with a standard deviation of 1.1 per cent.

The levels of plasma tryptophan of ten fasting normal subjects were determined, duplicate runs being made on each blood sample on 2 successive days. The mean value on the 1st day was 1.13 mg per 100 ml with an

average deviation of 0.04, on the 2nd day  $1.11 \pm 0.05$ . The average variation in the values obtained for individual samples was 4.7 per cent.

#### DISCUSSION

Fluorometric assay, when applicable, offers a number of advantages over other methods of assay. Its sensitivity is much higher than that of spectrophotometry and its specificity is greater since it is based on two spectral requirements, activation and emission.

The spectrophotofluorometric assay of tryptophan and tyrosine in protein hydrolysates is a rapid, simple, and specific one and yields values which agree with those obtained by other accepted procedures. The light scattering by suspended silicates formed from the action of alkali on glass during alkaline hydrolysis of proteins illustrates an important interference in this type of assay. Fortunately light scattering occurs at the wavelength of activation and may, therefore, be distinguished from fluorescence. When scattering is unduly large, it will be immediately evident upon inspection of the spectrum.

The procedure for determining tryptophan in plasma is obviously simpler than the bioassay methods now in use. The availability of spectrophotofluorometer type instruments should make plasma tryptophan a useful clinical assay. Preliminary studies in this laboratory indicate that, in patients with malignant carcinoid, levels of fasting plasma tryptophan are frequently below normal. This is consistent with the huge excretion of the tryptophan metabolite, 5-hydroxyindoleacetic acid, previously reported in patients with this disorder (7). Tryptophan tolerance studies in humans are also being carried out by using the spectrophotofluorometric assay.

#### SUMMARY

1. Ultraviolet fluorescent spectra of tryptophan and tyrosine have been used for the determination of these amino acids in protein hydrolysates.

2. A spectrophotofluorometric method for the determination of free tryptophan in plasma is described. Levels of plasma tryptophan in normal fasting subjects were determined and found to be in good agreement with the results of microbiological determinations cited by other workers.

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# THE INITIAL STEP IN ENZYMATIC SULFITE OXIDATION\*

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Previous studies have established the presence in liver of an enzyme system which catalyzes the oxidation of sulfite (1-4). Some of the properties of this system have been described (1, 2), as well as a procedure for its partial purification (4). The system is known to be a complex of several enzymes and involves the reversible participation of hypoxanthine or mosine (4) and a terminal flavoprotein. The present report is concerned with the initial event in this series of reactions.

## EXPERIMENTAL

The enzyme preparation used in these studies was identical with that employed previously and designated Fraction F-IIIa (4). Essentially, it was prepared by extraction of acetone-powdered dog liver with 10 volumes of 0.05 M phosphate buffer, pH 7.8, removal of the protein insoluble at pH 5.5, and that precipitated by brief exposure to 60°, and removal of nucleic acids with protamine. All assays were performed in the presence of 0.01 per cent Versene to prevent sulfite autooxidation. The preparation catalyzes aerobic sulfite oxidation in phosphate or tris(hydroxymethyl)-aminomethane buffer and also catalyzes reduction of methylene blue, gallocyanin, and other oxidation-reduction dyes.

The anaerobic reduction of methylene blue proved to be inhibited 50 per cent by  $10^{-4}$  M arsenite and by  $10^{-4}$  M *p*-chloromercuribenzoate (PCMB). This level of sensitivity is below that of the pyruvate and ketoglutarate oxidase systems in which lipoate is employed, but considerably above that of the numerous other enzymes which are sensitive to these agents at concentrations of 0.01 to 0.001 M. In contrast, the *aerobic* oxidation of sulfite was much less sensitive to arsenite, a concentration of 0.01 M was necessary to achieve 50 per cent inhibition.

It appeared, therefore, that in oxygen the sensitive sulfhydryl groups are maintained in the disulfide form and are less readily available to react with agents such as arsenite. The suggestion thus arose that sulfite oxidation

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may involve reversible reduction and oxidation of a disulfide structure. This was substantiated by the progressive nature of PCMB inhibition. Whereas preincubation with 0.0025 M PCMB was almost without effect, the aerobic oxidation of sulfite was inhibited 5 per cent in the first 10 minutes, 15 per cent in the second 10 minutes, and 40 per cent in the third 10 minutes. When samples of the enzyme preparation were anaerobically incubated with PCMB and examined spectrophotometrically by the Boyer procedure (5), the presence of sulfite (10  $\mu$ moles per ml, pH 7.0) elicited

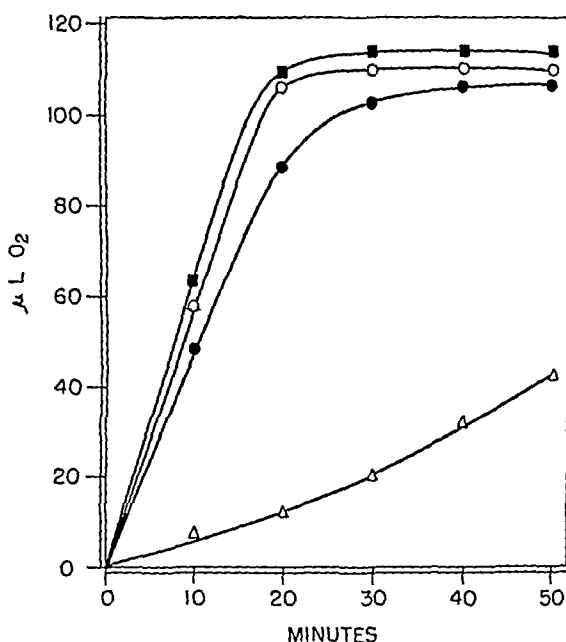


FIG. 1. Oxidation of sulfite and sulfhydryl compounds by partially purified sulfite oxidase. Each flask contained 1.0 ml of enzyme and one of the following: sulfite, 10  $\mu$ moles (●), cysteine, 20  $\mu$ moles (○), dimercaptolipoate, 10  $\mu$ moles (■), or reduced glutathione, 25  $\mu$ moles (Δ), in 2.2 ml of 0.05 M potassium phosphate, pH 7.8, containing 0.01 per cent Versene Fe-III.

the exposure of 0.9 to 2.2 moles of sulfhydryl per 100,000 gm of protein. Because of the crude state of the enzyme preparation, the absolute values here are without significance, but they are compatible with the possibility that sulfite oxidation may involve the reversible participation of disulfide and sulfhydryl groups.

This possibility was further supported by the observation that both dimercaptolipoate<sup>1</sup> and cysteine were oxidized by the same preparation,

<sup>1</sup> Dimercaptolipoate was prepared by reduction with sodium borohydride at pH 7, the excess borohydride was discharged with acid and the dimercaptan was extracted into benzene. Almost quantitative yields were obtained when the reaction was followed by observing the absorption of the disulfide form at 330 m $\mu$  (7) and

aerobically, at a rate comparable to that of sulfite (Fig 1) In contrast, the oxidation of reduced glutathione was relatively slow Further evidence was offered by the surprising observation that the enzyme system respired with borohydride as "substrate" The addition of 1.0  $\mu$ mole of oxidized lipoate to a vessel containing 20  $\mu$ moles of sulfite accelerated oxygen consumption by 15 to 40 per cent In contrast, an equivalent amount of cystine effected a 55 per cent decrease in the rate of sulfite oxidation, again suggesting that lipoate, rather than cystine, may be the source of the sulphydryl group involved in sulfite oxidation (Fig 2) When

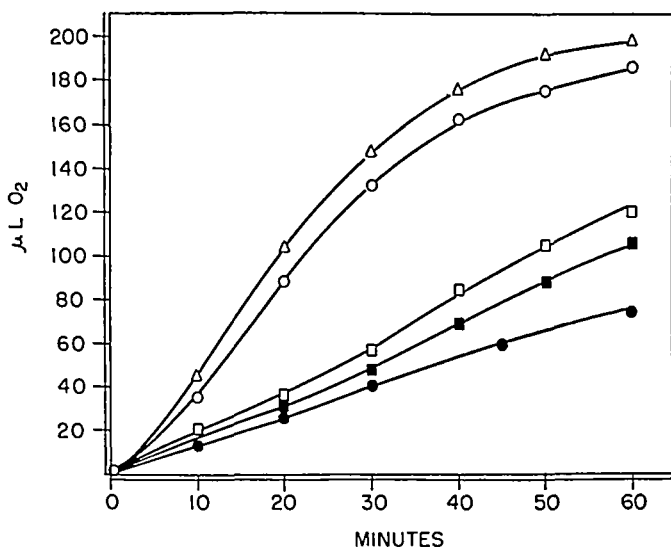


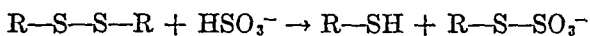
FIG 2 Effect of lipoate and various inhibitors on the oxidation of sulfite (O) Each flask contained 1.0 ml of enzyme, and 10  $\mu$ moles of sulfite in 2.2 ml of 0.05 M potassium phosphate, pH 7.8, containing 0.01 per cent Versene Fe-III. Appropriate flasks contained 1.0  $\mu$ mole of lipoate ( $\Delta$ ), 1.0  $\mu$ mole of cystine ( $\square$ ), 4  $\mu$ moles of cysteine thiosulfonate ( $\blacksquare$ ), or 4  $\mu$ moles of thiosulfate ( $\bullet$ )

the enzyme preparation was offered a mixture of sulfite (10  $\mu$ moles) and dimercaptolipoate (10  $\mu$ moles), the rate of oxidation was not significantly greater than that observed when the smaller dimercaptolipoate concentration was employed, thus indicating that the same reaction step is limiting in the enzymatic oxidation of these compounds. However, when cysteine and sulfite were similarly combined, the rate of oxygen consumption, while initially rapid, soon declined markedly, presumably because of the inhibitory effect of the cystine so formed.

measuring the sulphydryl produced by the Boyer procedure (5). Per sulphydryl released, the usual reaction with nitroprusside was about 10 per cent that with cysteine, presumably accounting for the statement that lipoate cannot readily be reduced by borohydride (7).

A series of sulfur-containing acids was tested as possible inhibitors of sulfite oxidation. Benzenesulfonic acid, pyridine-3-sulfonic acid, ethane and methanesulfonic acids, cysteic acid, and cysteinesulfonic acids were found to be without effect. Inorganic thiosulfate, however, proved to be an effective inhibitor of sulfite oxidation both aerobically and in the dye reduction assay (Fig 2).

The structure of thiosulfate,  $\text{S}-\text{SO}_3^-$ , suggested a possible mechanism for the initial step in sulfite oxidation. Bisulfite is known to react with disulfides, cleaving them to a sulfhydryl and thiosulfonate (6). Thus, were



$\text{HSO}_3^-$  to react with a disulfide component of the enzyme system, subsequent hydrolysis of the thiosulfonate would yield a second sulfhydryl group and sulfate. Were lipoate the disulfide in question, the mechanism might be depicted as shown in Fig 3.

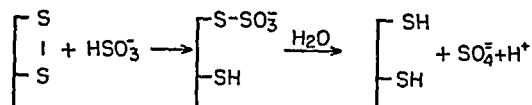


FIG 3 The initial steps in sulfite oxidation

Since cystine inhibited sulfite oxidation, cysteine thiosulfonate was prepared from cystine (6) and was found to inhibit sulfite oxidation, aerobically and in the dye reduction system, about as effectively as does thiosulfate (Fig 2). As neither thiosulfate nor cysteine thiosulfonate inhibited the oxidation of dimercaptolipoate or cysteine, it was concluded that these agents may inhibit sulfite oxidation by competing with the active thiosulfonate in the hydrolytic step of the sequence shown in Fig 3. Accordingly, it seemed desirable to determine whether the enzyme preparation was capable of catalyzing the hydrolysis and subsequent oxidation of lipoate hemithiosulfonate. However, several attempts to prepare this compound, by modifications of the procedure used to prepare cysteine thiosulfonate, failed. Similar results have been reported by Calvin and are in keeping with the unusual nature of lipoate as a disulfide (7). Indeed, since  $E'_0$  for the lipoate system is 0.2 volt below that of cysteine while  $E'_0$  for the oxidation of sulfite is of the same order as that of cysteine (8), it is unreasonable to expect significant net synthesis of lipoate hemithiosulfonate. This consideration also accounts for the fact that no significant appearance of  $-\text{SH}$  groups was detected when sulfite and oxidized lipoate in substrate amounts were incubated anaerobically with the enzyme preparation and the reaction followed at 330 m $\mu$ .

The sum of these observations, *viz* oxidation of cysteine and dimercapto-

lipoate, sensitivity of sulfite oxidation to aisenite and PCMB inhibition, catalysis of sulfite oxidation by lipoate but inhibition by cystine, and inhibition by cysteine thiosulfonate and by thiosulfate, affords strong evidence in support of the reaction sequence shown in Fig 3 and suggests but does not establish that lipoate is the disulfide compound involved

Sorbo (9) has postulated a mechanism for the action of rhodanese which involves addition of thiosulfate across an enzyme-bound disulfide group. The similarity of such a reaction with that proposed above, the inhibition of sulfite oxidation by thiosulfate, and the inhibition of rhodanese by sulfite reported by Sorbo led us to investigate the rhodanese activity of our enzyme preparations. Crude preparations of both rat and dog liver sulfite oxidase were rich in rhodanese. However, the final, partially purified preparations were practically devoid of rhodanese activity. Rhodanese and the sulfite-oxidizing system are, therefore, distinct entities.

#### SUMMARY

The oxidation of sulfite by a partially purified enzyme system from dog liver is inhibited by sulfhydryl reagents, thiosulfate, cysteine thiosulfonate, and cysteine but accelerated by lipoate. The preparation also oxidizes dimercaptolipoate and cysteine. It is concluded that the initial event in sulfite oxidation in this system is reaction with a disulfide to form a thio-sulfonate which is hydrolyzed to a sulfhydryl compound and sulfate. It is suggested that lipoate may be the source of the disulfide group.

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# ENZYMATIC SYNTHESIS OF ADENOSINE-5'-PHOSPHATE FROM INOSINE-5'-PHOSPHATE\*

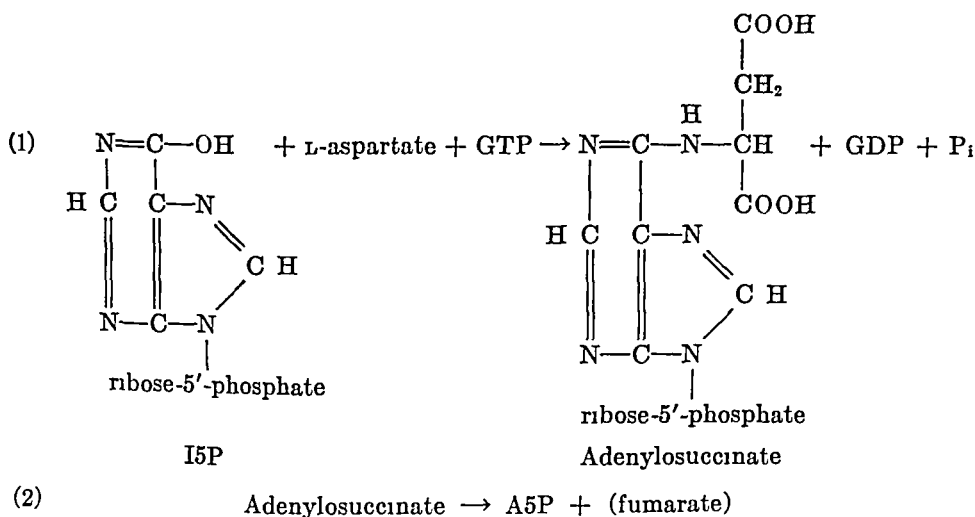
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(Received for publication, April 16, 1956)

Studies on the synthesis of cytidine nucleotides revealed that the enzyme is specific for uridine polyphosphate, and that  $\text{NH}_3$  is the direct source of the amino group (1, 2). It was of interest to investigate the amination of a purine nucleotide to determine whether the reactions are similar. In a preliminary report (3), evidence obtained with enzyme preparations from *Escherichia coli*, strain B, was presented for the synthesis of A5P<sup>1</sup> in a two-step reaction sequence involving I5P, not a nucleoside polyphosphate, and L-aspartate, not  $\text{NH}_3$ .



In the first step, I5P and L-aspartate are condensed to form adenylosuccinate.

\* This investigation was supported by a grant from the National Institutes of Health, Public Health Service

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<sup>1</sup> The abbreviations used are as follows: inosine-5'-phosphate, I5P, inosine diphosphate, IDP, inosine triphosphate, ITP, guanosine-5'-phosphate, G5P, guanosine diphosphate, GDP, guanosine triphosphate, GTP, adenosine-5'-phosphate, A5P, adenosine diphosphate, ADP, adenosine triphosphate, ATP, cytidine triphosphate, CTP, uridine triphosphate, UTP, inorganic orthophosphate, P<sub>i</sub>.



nate (equation (1)), a compound first synthesized by Carter and Cohen (4). This reaction is mediated by an enzyme called adenylosuccinate synthase. A5P is then formed by the cleavage of adenylosuccinate (equation (2)), a reaction first described by Carter and Cohen (4) with an enzyme from yeast. These results are in agreement with those of Abrams and Bentley (5) who observed the over-all reaction with rabbit bone marrow extracts.

The purpose of this report is to present in more detail the evidence for reactions (1) and (2) and to describe some of the properties of reaction (1).

### *Materials and Methods*

*Preparation of Cell-Free Extract*—*E. coli*, strain B, was grown on a glucose and inorganic salts medium<sup>2</sup> at 37° with vigorous shaking. When the optical density, measured with a Coleman junior spectrophotometer at 540 m $\mu$ , was approximately 0.7, the cultures were cooled, and the cells were collected by centrifugation at 3°. Cell-free extracts were prepared by grinding with alumina (Alcoa A-301, 2.5 gm per gm of packed, wet cells) (6) and extracting with potassium phosphate buffer (0.005 M, pH 7.2, 5.5 ml per gm of wet cells). Insoluble material was removed by centrifugation (approximately 10,000  $\times g$ ).

*Materials*—I5P and ATP were obtained from the Sigma Chemical Company as the crystalline sodium salts. GDP, GTP, UTP, IDP, and ITP were obtained from the same source and were further purified by anion exchange or paper chromatography. G5P, ADP (Sigma Chemical Company), and cytidine di- and triphosphate (Pabst Brewing Company) were used without further purification.

8-C<sup>14</sup>-A5P was synthesized enzymatically from radioactive adenine and 5'-phosphoribosyl pyrophosphate (7). 8-C<sup>14</sup>-I5P was prepared from the labeled A5P by deamination with Schmidt's deaminase. Concentration was effected by adsorption and elution from Norit. 6-O<sup>18</sup>-I5P was obtained by enzymatically deaminating A5P in H<sub>2</sub>O<sup>18</sup>. The reaction mixture (1.25 ml) contained 1.0 ml of H<sub>2</sub>O<sup>18</sup> (6.13 atom per cent excess), 0.15 ml of ammonium formate buffer (5 M, pH 5.9), 0.05 ml of A5P (0.645 M), and 0.05 ml of Schmidt's deaminase. When spectrophotometric examination of aliquots revealed that the reaction was complete, the deaminase was destroyed by heating in a boiling water bath for 2 minutes. Water and ammonium formate were removed by lyophilization.

The preparation and crystallization of 1,4-C<sup>14</sup>-L-aspartic acid were previously described (8). Phosphoenolpyruvic acid was prepared according

2.15 gm of KH<sub>2</sub>PO<sub>4</sub>, 13.5 gm of Na<sub>2</sub>HPO<sub>4</sub>, 0.2 gm of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 2.0 gm of NH<sub>4</sub>Cl, 10 mg of CaCl<sub>2</sub>, and 0.5 mg of FeSO<sub>4</sub> · 7H<sub>2</sub>O were dissolved in distilled water to 900 ml. After sterilization by autoclaving, 100 ml of a sterile 4 per cent solution of glucose were added.

to the procedure of Baer and Fischer (9) and purified by anion exchange chromatography (10)  $\text{NH}_2\text{OH}$  was prepared from  $(\text{NH}_2\text{OH})_2\text{SO}_4$  by treatment with solid  $\text{Ba}(\text{OH})_2$

Phosphopyruvate kinase was crystallized from rabbit muscle by the procedure of Beisenherz *et al* (11) and Schmidt's deaminase was prepared by the method of Kalckar (12) 5'-Nucleotidase, purified from bull semen by the procedure of Heppel and Hilmo (13), was a gift from Dr L. A. Heppel

*Determinations*—A5P was estimated with Schmidt's deaminase by Kalckar's method (14) Pentose was determined by the method of Mejbaum (15) and protein was determined by the procedure of Lowry *et al* (16) Orthophosphate was estimated according to Fiske and Subbarow (17), acid-labile phosphate was the orthophosphate liberated after hydrolysis for 12 minutes in 1 N  $\text{H}_2\text{SO}_4$  at  $100^\circ$ , and total phosphate was obtained as orthophosphate after ashing with an  $\text{H}_2\text{SO}_4\text{-HNO}_3$  mixture Radioactivity measurements were made with a gas flow counter after drying the samples in aluminum dishes

*Adenylosuccinate Synthase*—Adenylosuccinate synthesis (equation (1)) was measured in several ways Usually the enzyme was estimated with the Beckman DU spectrophotometer by the increase in optical density at  $280\text{ m}\mu$  in test mixtures wherein GTP was constantly regenerated from the GDP formed (Assay I) GTP regeneration occurred at the expense of ATP and was catalyzed by a nucleoside diphosphokinase (18) present as a contaminant in the enzyme preparation When it was desirable to avoid the requirement for GTP regeneration, a larger amount of GTP was added and ATP was omitted (Assay II) Assay I has the advantages of a lower blank absorption and of proportionality of adenylosuccinate synthesis over a wider range of enzyme concentrations

When compounds having high optical absorption at  $280\text{ m}\mu$  were tested or when greater sensitivity was required,  $\text{C}^{14}$ -L-aspartate was used in the assay mixture, and adenylosuccinate synthesis was measured as the radioactivity which remained on an anion exchange column after elution of the aspartic acid (Assay III)

*Assay I*—The reaction mixtures (0.7 ml) contained 0.1 ml of glycine buffer (1 M, pH 8.0), 0.04 ml of  $\text{MgCl}_2$  (0.1 M), 0.04 ml of ATP (0.001 M), 0.04 ml of phosphoenolpyruvate (0.01 M), 30 units of pyruvate phosphokinase, 0.05 ml of L-aspartate (0.01 M), 0.01 ml of GTP (0.001 M), 0.06 ml of I5P (0.005 M), and the enzyme preparation After 30 minutes at  $37^\circ$ , 0.5 ml of perchloric acid (7 per cent) was added and insoluble material was removed by centrifugation The increase in optical density ( $280\text{ m}\mu$ ) was determined by comparison with a control mixture to which one omitted component (I5P, GTP, aspartate, or enzyme) was added after the perchlo-

ric acid A unit of enzyme was defined as the amount yielding an increase in optical density of 0.100 during the test period Specific activity was defined as units of activity per mg. of protein

Under the conditions of Assay I, the rate of adenylosuccinate formation was proportional to the amount of enzyme over the range tested Thus, the number of enzyme units per ml. with 0.01, 0.02, 0.03, 0.04, 0.06, and 0.08 ml. of enzyme solution were 90.0, 92.0, 92.6, 94.0, 88.2, and 85.1, respectively The rate of reaction was essentially linear for 60 minutes Thus with a constant amount of enzyme, 74.4, 86.4, 82.8, 84.2, 80.0, and 75.8 units of enzyme per ml. were calculated to be present after incubation for 5, 10, 20, 30, 45, and 60 minutes, respectively

*Assay II*—The reaction mixtures (0.7 ml.) contained 0.1 ml. of glycine buffer (1 M, pH 8.0), 0.04 ml. of  $MgCl_2$  (0.1 M), 0.05 ml. of L-aspartate (0.01 M), 0.03 ml. of I5P (0.005 M), 0.10 ml. of GTP (0.001 M), and the enzyme preparation The conditions of incubation and spectrophotometric examination were the same as those for Assay I

Under the conditions of Assay II the rate of adenylosuccinate formation was proportional to the amount of enzyme over only a narrow range, 0 to 2 units Thus, the increase in optical density (280  $m\mu$ ) per ml. of enzyme solution in 30 minutes was calculated to be 12.6, 13.4, 12.5, 12.2, 10.9, and 9.4 with 0.005, 0.01, 0.015, 0.02, 0.03, and 0.04 ml. of enzyme, respectively A gradual decrease in reaction rate occurred with time

*Assay III*—The test mixtures (0.7 ml.) were the same as those for Assay II except that 0.06 ml. of 1,4- $C^{14}$ -L-aspartate (0.0056 M, 590,000 c.p.m. per  $\mu$ mole) was substituted for the unlabeled amino acid and a larger amount of enzyme was used (about 10 units) After 30 minutes at 37° the reaction was stopped by heating in a boiling water bath for 2 minutes and 2 ml. of water were added to increase the volume Insoluble material was discarded by centrifugation and the supernatant solutions were chromatographed on columns of Dowex 1, chloride form (2 per cent cross-linked, height 2 cm., diameter 1 cm.), at 3° Aspartic acid was eluted with 100 ml. of 0.01 N HCl, adenylosuccinic acid with 5 ml. of 1 N HCl Radioactivity measurements were made on aliquots (0.2 ml.) of the 1 N HCl eluate and a self-absorption correction factor of 2.25 was applied

### Results

*Purification of Adenylosuccinate Synthase*—Purification of the enzyme was carried out at 0–3° unless otherwise indicated Recoveries of activity greater than 100 per cent are considered to result in large part from the removal of the adenylosuccinase activity (equation (2))

*Treatment with Streptomycin*—To 50 ml. of cell-free extract 13 ml. of a 5 per cent solution of streptomycin sulfate (Merck) were added with stir

ring After 5 minutes the stinging precipitate was discarded by centrifugation (streptomycin fraction, Table I)

*Low pH Treatment I*—The pH of the streptomycin fraction (56 ml) was adjusted to 5.4 by the addition of 0.1 M acetic acid (6 to 7.5 ml) with stirring. The solution was kept in a water bath at 37° for 10 to 15 minutes with constant stirring. It was then cooled and the supernatant solution, obtained by centrifugation, was neutralized with 1 M KOH (low pH fraction I, Table I)

*Precipitation with Ammonium Sulfate*—To the low pH fraction I (60 ml) 30 ml of glycine buffer (1 M, pH 9.6) followed by 30.9 gm of ammonium sulfate were added with stirring. After 5 minutes, the precipitate was discarded by centrifugation and an additional 8.4 gm of ammonium sulfate

TABLE I  
*Purification of Adenylosuccinate Synthase*

Enzyme fraction	Volume of solution	Total units*	Total protein	Specific activity
	ml <sup>a</sup>		mg	units per mg protein
Cell-free extract	50	2200	590	3.7
Streptomycin	56	3920	353	11.1
Low pH I	60	7100	226	31.4
Ammonium sulfate	40	4240	53	80.0
Low pH II	20	2060	9.0	229

\* The low recovery of activity in the more crude preparations is considered to be due to the further metabolism of some of the adenylosuccinate formed

were added to the supernatant fluid. The precipitate, collected after 5 minutes, was dissolved in water to a volume of 40 ml (ammonium sulfate fraction, Table I)

*Low pH Treatment II*—0.1 M acetic acid (2.5 to 3.5 ml) was slowly added to the ammonium sulfate fraction with stirring to lower the pH to 4.3. After 10 minutes, the precipitate was obtained by centrifugation and dissolved in glycine buffer (0.05 M, pH 8.5) to a volume of 20 ml (low pH fraction II, Table I). The experiments reported here were carried out with the low pH fraction II which was free of adenylosuccinase activity.

### *Stoichiometry of Reaction*

The stoichiometry of adenylosuccinate formation (equation (1)) was studied with C<sup>14</sup>-labeled aspartate (Table II). Anion exchange chromatographic analysis revealed that the condensation of I5P and L-aspartate was accompanied by an equivalent disappearance of GTP and appearance of GDP and P<sub>i</sub>. I5P was identified by its chromatographic behavior and

by its absorption spectrum (peak at 249  $m\mu$ ,  $\lambda_{250}/\lambda_{260} = 1.60$ ,  $\lambda_{280}/\lambda_{260} = 0.21$ , at pH 2), and GDP and GTP by their chromatographic properties and absorption spectrum (peak at 256  $m\mu$ ,  $\lambda_{250}/\lambda_{260} = 0.99$ ,  $\lambda_{280}/\lambda_{260} = 0.68$ , at pH 2). GDP and GTP were further characterized by their molar ratios of guanine, pentose, acid-labile P, and total P of 1.00 1.02 1.02 2.01 and 1.00 0.94 1.94 2.90, respectively. Adenylosuccinate was identified by its

TABLE II

*Stoichiometry of Adenylosuccinate Synthesis*

The reaction mixture (29.4 ml) contained 4.2 ml of glycine buffer (1 M, pH 8.0), 1.68 ml of  $MgCl_2$  (0.1 M), 1.68 ml of  $C^{14}$ -carboxyl-labeled L-aspartate (0.01 M, 147,000 c.p.m. per  $\mu$ mole), 0.85 ml of I5P (0.01 M), 1.42 ml of GTP (0.0059 M), and 416 unit of adenylosuccinate synthase (specific activity 225). An aliquot of the reaction mixture (15 ml) was placed immediately in a boiling water bath for 2.5 minutes, the remainder was incubated at 37° for 50 minutes and then heated for 2.5 minutes in a boiling water bath. Aliquots (12 ml) of the reaction mixtures were chromatographed on columns of Dowex 1, chloride form (2 per cent cross-linked, height 5 cm, diameter 1 cm), at 3°. Aspartic acid and I5P were eluted separately with a solution of 0.01 N HCl, adenylosuccinate, with 0.01 N HCl-0.025 M KCl, GDP, with 0.01 N HCl-0.05 M KCl, and GTP, with 0.02 N HCl-0.2 M KCl. Aspartic acid was estimated by radioactivity measurements, I5P, adenylosuccinate, and the guanosine nucleotides by spectrophotometric measurements at 250, 267, and 260  $m\mu$ , respectively.  $P_i$  was estimated before chromatography according to Fiske and Subbarow (17).

	0 min	50 min	$\Delta$		Specific activity
	$\mu$ moles	$\mu$ moles	$\mu$ moles	total c.p.m.	c.p.m. per $\mu$ mole
I5P	3.43	1.75	-1.68	0	
GTP	3.39	1.63	-1.76	0	
L-Aspartate	6.87	5.21	-1.66	-244,020	147,000
Adenylosuccinate	0.00	1.61*	+1.61	+227,180	141,100
GDP	0.00	1.72	+1.72	0	
$P_i$	0.39	2.08	+1.69		

\* A value of 1.66  $\mu$ moles was obtained for adenylosuccinate production by optical density measurements at 280  $m\mu$  before chromatography.

absorption spectrum (4) (peak at 267  $m\mu$ ,  $\lambda_{250}/\lambda_{260} = 0.64$ ,  $\lambda_{280}/\lambda_{260} = 0.68$ , at pH 2, peak at 269  $m\mu$ ,  $\lambda_{250}/\lambda_{260} = 0.60$ ,  $\lambda_{280}/\lambda_{260} = 0.81$ , at pH 12) and by the molar ratios ( $E_m$  267  $m\mu$  at pH 1 =  $16.9 \times 10^3$  (4)) of pentose and total P of 0.99 and 0.96, respectively. No detectable  $P_i$  was liberated during incubation in 1 N  $H_2SO_4$  in a boiling water bath for 15 minutes, but, from 0.107  $\mu$ mole, 0.109  $\mu$ mole of  $P_i$  was released by 5'-nucleotidase (13). As can be seen from Table II, the specific activity of the adenylosuccinate (141,100 c.p.m. per  $\mu$ mole) was essentially the same as that of the L-aspartate.

### *Specificity of Substrates*

*I5P*—When hypoxanthine or inosine was substituted for I5P, no detectable synthesis of adenylosuccinate occurred. With IDP and ITP, the reaction rate was 19.8 and 5.7 per cent of that with I5P, respectively. The activity of the inosine polyphosphates may result from their conversion to I5P.

The ability of 5'-phosphoribosyl-5-amino-4-imidazolecarboxamide<sup>3</sup> to replace I5P was tested with radioactive L-aspartate (Assay III). With no additions, 0.1  $\mu$ mole of the aminoimidazolecarboxamide ribotide, and 0.1  $\mu$ mole of I5P, 0, 68, and 40,600 c.p.m., respectively, were found in the adenylosuccinate area. The acyclic compound did not inhibit the reaction with I5P, a test mixture containing both nucleotides yielded 38,900 c.p.m. in the adenylosuccinate area.

*L-Aspartate*—The following compounds were found to be inactive:  $\text{NH}_3$  (0.01 M), L-asparagine, D-aspartate, L-glutamate, L-glutamine,  $\beta$ -alanine (each  $7 \times 10^{-4}$  M), and DL- $\alpha$ -alanine ( $1.4 \times 10^{-3}$  M). Inhibition of adenylosuccinate synthesis was absent or was less than 10 per cent.

*GTP*—No adenylosuccinate formation occurred in the absence of GTP. By using Assay I, the following compounds were found to be totally incapable of replacing GTP: A5P, G5P, GDP, and the di- and triphosphates of adenosine, cytidine, uridine, and inosine. The nucleoside triphosphates (each 0.1  $\mu$ mole) were also tested with the more sensitive chromatographic assay (Assay III) with the following results: none, 0, ATP, 0, CTP, 0, UTP, 295, ITP, 270, GTP, 39,500 c.p.m. of adenylosuccinate. The activity observed with UTP and ITP may be due to their contamination with GTP.

### *Effect of pH and Other Factors on Adenylosuccinate Synthesis*

The effect of pH and other factors was measured with the spectrophotometric assay involving no regeneration of GTP (Assay II).

*pH*—The rate of adenylosuccinate synthesis was studied as a function of pH with glycine and orthophosphate buffers. With the glycine buffers the optimal pH range was 7.3 to 7.8. At pH 6.6, 8.4, and 9.2 the reaction rates were 63, 51, and 0 per cent, respectively, of the rate in the optimal range. With the phosphate buffers (0.07 M) the optimal range was pH 7 to 8, but the maximal rate of adenylosuccinate synthesis was only 80 per cent of that with glycine buffer.

*Mg<sup>++</sup>*—In the absence of added  $\text{Mg}^{++}$  no adenylosuccinate synthesis occurred. The reaction rate was improved by increasing the  $\text{Mg}^{++}$  concentration up to 0.003 M as follows: at 0, 3.8, 7.5, 15, 30, and  $60 \times 10^{-4}$  M  $\text{Mg}^{++}$ , the optical density increase (280  $\text{m}\mu$ ) was, respectively, 0.000,

<sup>3</sup> Kindly supplied by Dr. G. R. Greenberg.

0.060, 0.122, 0.178, 0.236, and 0.242. The  $Mg^{++}$  requirement could not be satisfied by  $Co^{++}$  or  $Zn^{++}$ , but with  $Mn^{++}$  and  $Ca^{++}$  (each 0.006 M) the rate of adenylosuccinate synthesis was 64 and 40 per cent, respectively, of that with  $Mg^{++}$  (0.006 M).

*Inhibitors*—Approximately 50 per cent inhibition of adenylosuccinate synthesis occurred with  $10^{-4}$  M GDP, 0.01 M glycylglycine buffer (pH 7.5), 0.08 M tris(hydroxymethyl)aminomethane buffer (pH 7.5), and 0.03 M NaF. The inhibitory action of GDP explains, at least in part, the gradual decrease in the rate of adenylosuccinate synthesis encountered with Assay II. Un

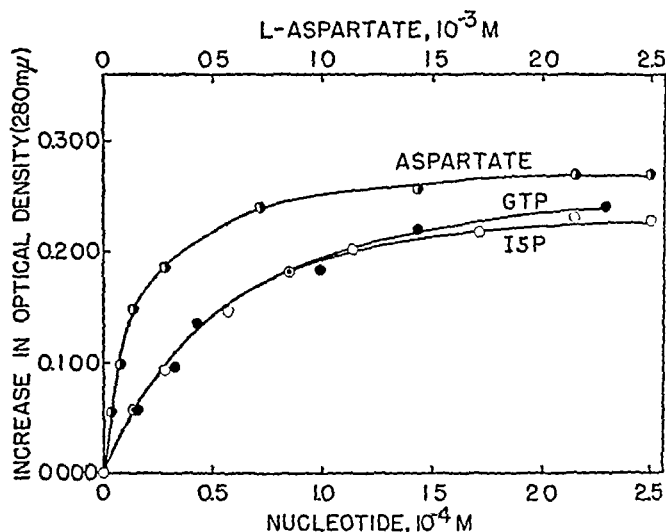


FIG. 1 The rate of adenylosuccinate synthesis as a function of substrate concentration. The reaction mixtures containing 2.0 units of adenylosuccinate synthase (specific activity 237) were prepared as for Assay II except that varying amounts of L-aspartate, GTP, or I5P were used. The rate of adenylosuccinate synthesis was estimated by the increase in optical density at 280  $m\mu$  after 30 minutes.

der the conditions of this assay an accumulation of GDP occurs. Inhibition by glycylglycine buffer could be almost completely reversed by increasing the  $Mg^{++}$  level. Thus, with 3, 6, 15, and 30  $\times 10^{-3}$  M  $Mg^{++}$ , the per cent inhibition with 0.007 M glycylglycine was 61, 39, 18, and 14, respectively. Acetylglutamine, DL-alanylglycine, and DL-alanylglycylglycine (each 0.014 M) were not inhibitory.

*Concentration of Substrates*—The influence of the concentrations of I5P, GTP, and L-aspartate on the reaction rate is shown in Fig. 1. The levels at which the rates were approximately half maximal were  $3 \times 10^{-5}$  M,  $4 \times 10^{-5}$  M, and  $1 \times 10^{-4}$  M for I5P, GTP, and L-aspartate, respectively.

#### Reversibility of Adenylosuccinate Synthase Reaction

The reversal of the synthase reaction should lead to the incorporation of P<sub>i</sub> into GTP (equation (1)). To test for reversibility, a reaction mixture

(1.03 ml) was prepared containing 100  $\mu$ moles of glycine buffer (pH 8.0), 4  $\mu$ moles of  $\text{MgCl}_2$ , 0.5  $\mu$ mole of GDP, 0.5  $\mu$ mole of adenylosuccinate, 10  $\mu$ moles of  $\text{P}_i^{32}$  ( $6.3 \times 10^5$  c.p.m. per  $\mu$ mole), and 20.2 units of adenylosuccinate synthase (specific activity 219). After incubation at  $37^\circ$  for 60 minutes the mixture was heated in a boiling water bath for 2 minutes, 1.5  $\mu$ moles of unlabeled GTP were added, and the solution was chromatographed on a column of Dowex 1, chloride form (2 per cent cross-linked, height 3 cm, diameter 1 cm), at  $3^\circ$ .  $\text{P}_i$ , adenylosuccinate, and GDP were washed off the column with 105 resin bed volumes of a solution of 0.01 N HCl-0.05 M KCl. GTP, recognized by its chromatographic behavior and absorption spectrum, was eluted with a solution of 0.02 N HCl-0.2 M KCl and contained 39,086 c.p.m. The specific activity of the six fractions which contained 96 per cent of the optical density units and 95.5 per cent of the radioactivity in the GTP area ranged from 25,300 to 26,700 c.p.m. per  $\mu$ mole. When adenylosuccinate was omitted from the test mixture, only 2101 c.p.m. were found in the GTP area.

### *Reaction with $\text{NH}_2\text{OH}$*

When  $\text{NH}_2\text{OH}$  replaced L-aspartate, a product was formed which was detected by an increase in optical density at  $280\text{ m}\mu$ . Substrate requirements for its formation were the same as those for adenylosuccinate synthesis. Thus, the increases in optical density with no GTP, I5P, or enzyme and with the complete system were 0.020, 0.003, 0.000, and 0.272, respectively. With no  $\text{NH}_2\text{OH}$ , the increase was 0.011.

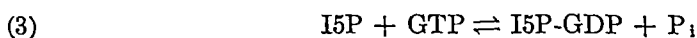
To obtain a sufficient amount of the product for its characterization, a test mixture (14.0 ml) was prepared containing 2 mmoles of glycine buffer (pH 8.0), 80  $\mu$ moles of  $\text{MgCl}_2$ , 1.2 mmoles of  $\text{NH}_2\text{OH}$  (pH 7.0), 10  $\mu$ moles of  $\text{C}^{14}$ -I5P (92,400 c.p.m. per  $\mu$ mole), 1  $\mu$ mole of GTP, and 388 units of adenylosuccinate synthase (specific activity 216). After 90 minutes at  $37^\circ$  the enzyme was destroyed by heating and the mixture was chromatographed on a column of Dowex 1, chloride form (2 per cent cross-linked, height 3 cm, diameter 1 cm) at  $3^\circ$ . A new compound (11.9 density units at  $265\text{ m}\mu$ ,  $\lambda_{280}/\lambda_{260} = 0.59$ ,  $\lambda_{250}/\lambda_{260} = 0.72$ , peak at  $265\text{ m}\mu$ , at pH 2) appeared between 2 and 5 resin bed volumes of 0.01 N HCl. The origin of the compound from I5P was indicated by its specific activity (93,150 c.p.m. per  $\mu$ mole, assuming  $E_m$   $265\text{ m}\mu$  at pH 2 = 16) and by the molar ratios of purine base, pentose, and 5'-nucleotidase-hydrolyzable P of 1.00:1.05:0.94. Paper chromatography of the compound (solvent = 57 parts of isobutyric acid, 4 parts of concentrated  $\text{NH}_4\text{OH}$ , and 39 parts of water) revealed a single ultraviolet-absorbing spot ( $R_f = 0.41$ ) which yielded a chromogen with acid- $\text{FeCl}_3$  solution (19), the color formed was blue. The compound has been tentatively identified as 6-N-hydroxy-A5P.



*Mechanism of Adenylosuccinate Synthase Reaction*

When GTP, I5P, or L-aspartate was omitted, chromatographic analysis carried out at pH 7 or 8 at 3° failed to reveal the accumulation of any new compounds in reaction mixtures containing either radioactive I5P (536,000 c p m per  $\mu$ mole) or L-aspartate (590,000 c p m per  $\mu$ mole)

The possibility was considered that an activated intermediate composed of a complex of GDP with I5P or L-aspartate is formed but does not accumulate. In this case, as illustrated for the GDP-I5P complex (equation (3)), the incorporation of  $P_i^{32}$  into GTP might be expected to occur in the absence of aspartate. The failure to find any difference in Nonit-adsorbable  $P^{32}$  with test mixtures lacking I5P, L-aspartate, or enzyme did not support this hypothesis



Evidence consistent with the formation of a phosphorylated intermediate was obtained in experiments with  $O^{18}$ . In one experiment, the test mixture was prepared exactly as described in Table II except that the I5P contained  $O^{18}$  on carbon atom 6. After 60 minutes at 37° the enzyme was destroyed by heating in a boiling water bath for 2 minutes.  $P_i$  (free of aspartate and I5P), GDP, and GTP were isolated by anion exchange chromatography. The fractions containing the guanosine nucleotides were combined, water was removed by lyophilization, and the mixture was hydrolyzed by heating for 15 minutes at 100° in 1 N HCl.

Analysis of the oxygen derived from the  $P_i$  and from the guanosine nucleotide phosphates yielded the following results:  $P_i$ , 4.88, nucleotide  $P_i$ , 0.00, I5P, 4.90 atom per cent excess  $O^{18}$ .

*Formation of A5P from Adenylosuccinate*

To study its further metabolism, 0.48  $\mu$ mole of adenylosuccinate labeled with  $C^{14}$  in the ring (specific activity 20,450 c p m per  $\mu$ mole) was incubated with a cell-free extract from *E. coli*. Anion exchange chromatography of the deproteinized reaction mixture revealed the formation of 0.46  $\mu$ mole of A5P (specific activity 19,420 c p m per  $\mu$ mole). The A5P was recognized by its chromatographic behavior (eluted from Dowex 1, 2 per cent cross linked, between 1.5 and 5.5 resin bed volumes of 0.01 N HCl, peak at 3.3 resin bed volumes) and by its absorption spectrum (peak at 258  $m\mu$ ,  $\lambda_{50}/\lambda_{258} = 0.82$ ,  $\lambda_{280}/\lambda_{260} = 0.23$ , at pH 2). Further characterization was obtained by conversion with Schmidt's deaminase to a compound having the absorption spectrum of inosine. I5P (0.45  $\mu$ mole, specific activity 19,460 c p m per  $\mu$ mole) was isolated by chromatography on Dowex 1 (eluted between

<sup>4</sup> The value for  $P_i$  has been corrected for dilution by the unlabeled oxygen atom of  $H_2PO_4$ , the value for the labeled oxygen of I5P was calculated

3 and 10 resin bed volumes of 0.05 N HCl, peak at 7 resin bed volumes of eluent)

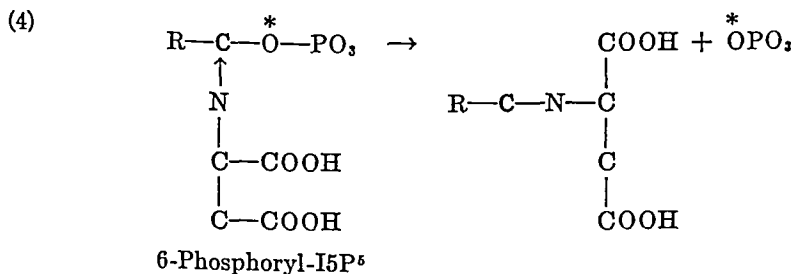
### DISCUSSION

The synthesis of adenylosuccinate as an intermediate in the conversion of I5P to A5P provides an important role for this compound. The possibility that other pathways for adenylosuccinate synthesis exist has been considered. One such pathway might involve the condensation of aspartate with 5-amino-4-imidazolecarboxamide ribotide followed by ring closure. With the enzyme from *E. coli* no evidence was obtained for a loss of carbon atom 2 from I5P. Thus, formate was not required for adenylosuccinate synthesis,  $C^{14}$ -formate was not incorporated into adenylosuccinate, no product possessing a diazotizable amine (20) accumulated, and the carboxamide ribotide appeared to be non-reactive in the synthesis of adenylosuccinate.

An interesting feature of the adenylosuccinate synthase reaction is the requirement for GTP. In only two other defined systems (exclusive of nucleoside mono- and diphosphokinase systems) has GTP been shown to serve as a phosphate donor in the synthesis of succinyl coenzyme A (21) and with chicken liver oxalacetate carboxylase (22).

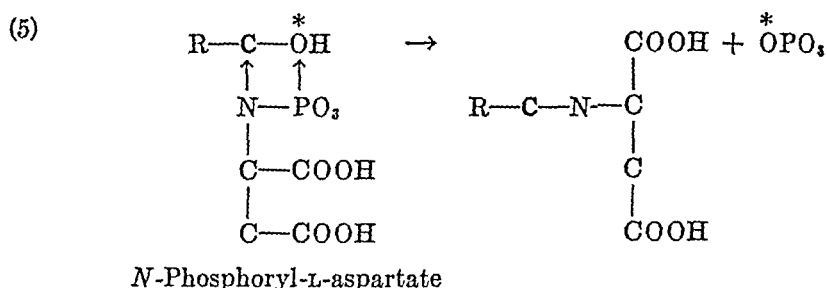
With regard to the mechanism of energy transfer from GTP, two types of intermediates were considered: GDP-substrate and phosphoryl-substrate. The GDP-substrate hypothesis was weakened by the failure to observe any incorporation of P<sub>i</sub> into GTP in the absence of either aspartate or I5P. It was further weakened when the transfer of O<sup>18</sup> from the 6 position of I5P to P<sub>i</sub> was observed during the condensation of I5P and L-aspartate. Formation of a GDP-aspartate or GDP-I5P complex cannot account for the observed fate of the oxygen atom without invoking complicated reaction mechanisms.

Of the two possible phosphoryl-substrate complexes, only 6-phosphoryl-I5P could yield adenylosuccinate by the simple mechanism of a nucleophilic attack (23) on carbon atom 6 of I5P (equation (4)). The synthesis of adenylosuccinate from *N*-phosphoryl-L-aspartate would involve a more



<sup>6</sup> R represents I5P exclusive of carbon atom 6 which is shown as C

complicated mechanism, a simultaneous double displacement reaction (equation (5))



The author wishes to thank Dr George Drysdale for carrying out the  $\text{O}^{18}$  determinations and for many helpful discussions

#### SUMMARY

1 An enzyme named adenylosuccinate synthase, partially purified from *Escherichia coli*, strain B, catalyzes the reaction

Inosine-5'-phosphate (I5P) + L-aspartate + guanosine triphosphate (GTP) → adenylosuccinate + guanosine diphosphate (GDP) + inorganic orthophosphate

2 Adenylosuccinate is cleaved by cell-free extracts to yield adenosine 5'-phosphate

3 The requirement for GTP for adenylosuccinate synthesis could not be satisfied by GDP or by the triphosphates of adenosine or cytidine. With uridine and inosine triphosphate the rate of the reaction was less than 1 per cent of that with GTP

4 The results of experiments with 6- $\text{O}^{18}$ -labeled I5P suggest the intermediary synthesis of 6-phosphoryl-I5P and the formation of adenylosuccinate by the displacement of the phosphate group by L-aspartate

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# ON THE MODE OF ACTION OF X-RAY PROTECTIVE AGENTS

## I THE FIXATION IN VIVO OF CYSTAMINE AND CYSTEAMINE TO PROTEINS\*

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(Received for publication, February 6, 1956)

Several hypotheses have been advanced in attempts to account for the ability of certain thiol (SH)- or disulfide (SS)-containing compounds to reduce the biological lesions caused by ionizing radiation (1, 2). It is clear that, in pure aqueous solutions, radiochemical transformations are for the greater part mediated by products formed by the interaction of ionizing radiation with water molecules (3). At the present time the favored hypothesis seems to be that these protective agents exert their action by reducing the number of radiation-induced free radicals (1, 2, 4). However, we have recently found that when cystamine, one of the most potent protective agents (5, 6), is present in a biological medium, its ability to inactivate free radicals is far too low to account for its protective ability *in vivo* (7). A reinvestigation of the detailed biochemistry of cystamine was therefore deemed desirable.

In the present paper, the metabolism of sulfur-labeled cystamine and cysteamine has been studied by means of methods which permitted the detection of  $10^{-7}$  mg of cystamine or cysteamine sulfur. It has been established that, after intraperitoneal administration of protective doses to mice, by far the greater part of the cystamine or cysteamine occurs in chemical combination with proteins and other constituents in serum and in the red cells. During the period of optimal protection only a minor fraction of cystamine or cysteamine is present in free form. It is suggested that this extensive, but temporary, binding of cystamine or cysteamine to tissue constituents is causally related to the protective ability of these compounds. Evidence is presented that the fixation is due to the formation of mixed disulfides between cysteamine and the SH groups of body constituents, a phenomenon which appears to be of general biochemical interest.

### *Materials and Methods*

Cystamine-S<sup>35</sup> dihydrochloride was synthesized (8, 9) with a specific activity of 3.5 to 6 mc per mg of S, giving under our conditions of measure-

\* Supported by grants from The Norwegian Cancer Society and from The Norwegian Research Council for Science and the Humanities.

ment  $0.74$  to  $1.27 \times 10^5$  c p m per  $\gamma$  of sulfur when counted on paper strips (Whatman No 1)

Labeled cysteamine hydrochloride was prepared by electrolytic reduction of labeled cystamine dihydrochloride by a procedure similar to the one described for the reduction of glutathione (10). 40 mg of cystamine  $S^{35}$  dihydrochloride, dissolved in 0.5 ml of phosphate buffer (0.067 M, pH 2.5) on top of a mercury surface of  $1.13 \text{ cm}^2$ , were subjected to a direct current of 3.54 ma per  $\text{cm}^2$ . The reduction under these conditions, as determined by iodometry, was found to be completed after 20 minutes. Paper chromatographic analysis established that cysteamine was the only labeled product.

Male adult mice (body weight 24 to 30 gm) were used in the experiments. The labeled compounds were given by intraperitoneal injection. The doses used (1.6 to 4.0 mg) were of the same magnitude as those shown to afford protection against ionizing radiation. After the desired time intervals, blood samples (5 to 10 mg) from a cut in the tail were drawn into heparinized capillaries. One end of the capillary was carefully sealed, and the blood corpuscles were separated from the serum by centrifugation. The hematocrit value was measured on the centrifuged capillaries.

Serum and blood corpuscles were obtained by dividing the capillary at the interface. Weighed amounts of serum and blood corpuscles were taken for determination of their total radioactivity. The isolated blood corpuscles were diluted 1:10 with physiological saline, hemolyzed by the addition of saponin, and centrifuged free of cell debris. The clear hemolysate and the serum samples were analyzed as described below.

The proteins of the serum samples were separated by paper electrophoresis on Whatman No 1 paper strips (width 1.8 cm) in Veronal buffer at pH 8.6 (ionic strength 0.1, 0.5 ma per strip, 18 hours). At alkaline reaction cystamine and cysteamine carry a positive charge and migrate in the direction opposite to the proteins. The paper in which the proteins migrate is therefore uncontaminated by radioactivity. The hemoglobin was isolated by paper electrophoresis under identical conditions, except that glycine buffer at pH 9.5 was used. The radioactive compounds were located and measured by means of a strip counter. The proteins on the electrophoretograms were subsequently stained by Amido black 10B and their relative amounts were determined colorimetrically according to Grassmann and Hamng (11).

Aliquots of serum and the red cell hemolysate were also analyzed by descending paper chromatography. Whatman No 1 paper strips, treated with 1 M KCl and 0.067 M sodium phosphate buffer at pH 7.4, were used. The solvent consisted of equal parts of isopropanol and ethanol with water added to a final concentration of 15 per cent. The same mixture was

placed in the bottom of the chromatography tank. In this system cystamine as well as microamounts of cysteamine appears at  $R_F$  0.40. When larger amounts of cysteamine were present, a broad peak with  $R_F$  0.53 appeared in front of the main peak at  $R_F$  0.40 (Fig. 6, C), possibly because of incomplete oxidation of the cysteamine (12). Taurine appears at  $R_F$  0.17, hypotaurine (2-aminoethanesulfonic acid) at  $R_F$  0.25 (12),<sup>1</sup> and sulfate at  $R_F$  0.0.

At the end of the experiments, the blood was collected by heart puncture. The total blood volume of the animal was assumed to be 7 per cent of the body weight.

Aliquots and dilutions were determined by weight. The specific gravities of whole blood, blood corpuscles, and serum were taken as 1.06, 1.09, and 1.03, respectively.

### Results

*Blood Radioactivity Distribution, Time Studies, Cystamine Experiments*—The experiment to be described below is one of three experiments which gave nearly identical results.

A male white mouse, weighing 28 gm, was injected intraperitoneally with 2.30 mg of cystamine- $S^{35}$  dihydrochloride. Blood samples were collected at intervals after the injection.

Typical examples of the protein radioactivity patterns obtained after paper electrophoresis are presented in Figs. 1 and 2. It is apparent that substantial amounts of radioactivity were bound to the hemoglobin and to the various serum proteins. The quantities of  $S^{35}$  found in the various blood fractions (serum albumin, total serum protein, total serum, hemoglobin, and whole blood) are presented in Fig. 3. The radioactivity of each fraction has been calculated for the total blood volume and is expressed as per cent of the injected dose. It appears that, during the 1st hour after the administration, the radioactivity is divided between serum and red cells roughly corresponding to the hematocrit value, while, at later times, a relatively greater part is found in the serum. The striking fact is that, during the period of maximal protection (10 to 45 minutes), the protein-bound radioactivity, in serum as well as in red cells, constitutes a considerable fraction of the total radioactivity. The fraction of the radioactivity bound to the proteins appears to be maximal within the first 15 minutes and subsequently decreases.

The paper chromatograms of the serum samples (Fig. 4) demonstrated the presence of cystamine or cysteamine and their known metabolites hypotaurine and taurine (13, 14). In order to obtain a complete separa-

<sup>1</sup> Since under our conditions the  $R_F$  of hypotaurine varies from 0.20 to 0.27 and that of taurine from 0.11 to 0.18, reference compounds were always run simultaneously.



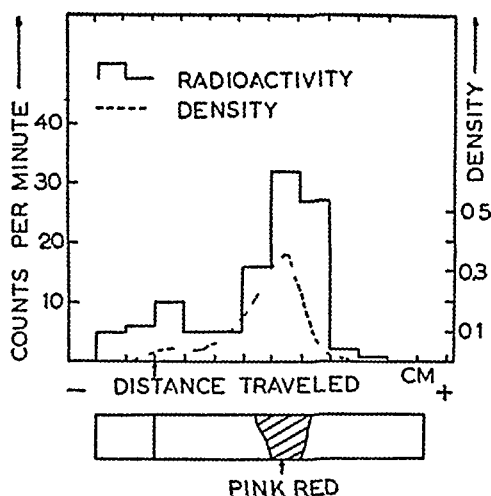


FIG 1 The binding of cystamine radioactivity to hemoglobin. The red cell collected 30 minutes after intraperitoneal administration of 2.3 mg of cystamine- $S^{35}$  to a mouse weighing 28 gm, were hemolyzed and subjected to paper electrophoresis in glycine buffer at pH 9.5. The radioactivity was measured in a strip counter and the protein pattern obtained was measured colorimetrically after staining with Amidoblack 10B. For further details see the text.

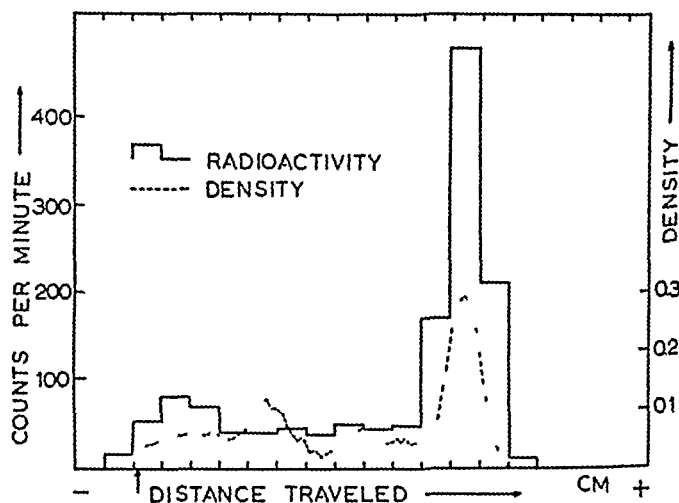


FIG 2 The binding of cystamine radioactivity to serum proteins. The serum sample was collected 30 minutes after intraperitoneal administration of 2.3 mg of cystamine- $S^{35}$  to a mouse weighing 28 gm and subjected to paper electrophoresis in Veronal buffer, pH 8.6. For further details see the text.

tion of these compounds, the chromatograms were run for an additional 12 hours after the front had left the paper. In Fig 4 the amount of each constituent is expressed as micrograms of the labeled sulfur injected found per ml of serum, permitting direct comparisons of the amounts present at different times. It is apparent that significant amounts of hypotaunne

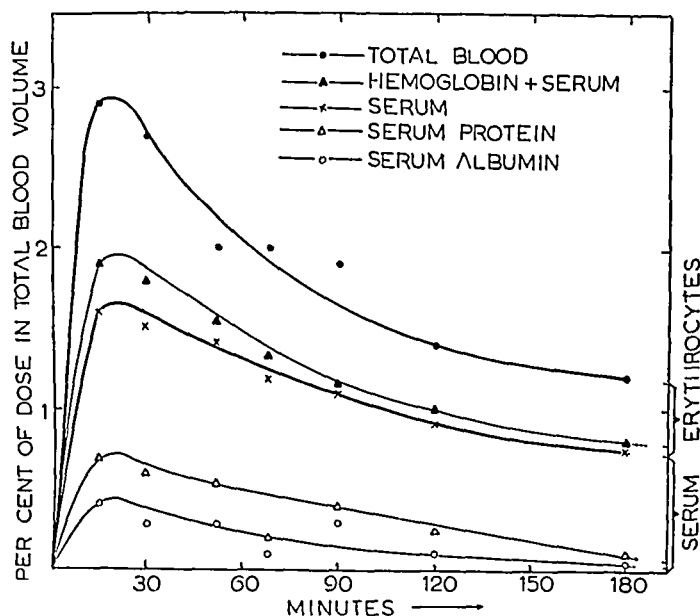


Fig 3 The  $S^{35}$  distribution among certain blood fractions at various times after intraperitoneal administration of 2.3 mg of cystamine- $S^{35}$  to a mouse weighing 28 gm

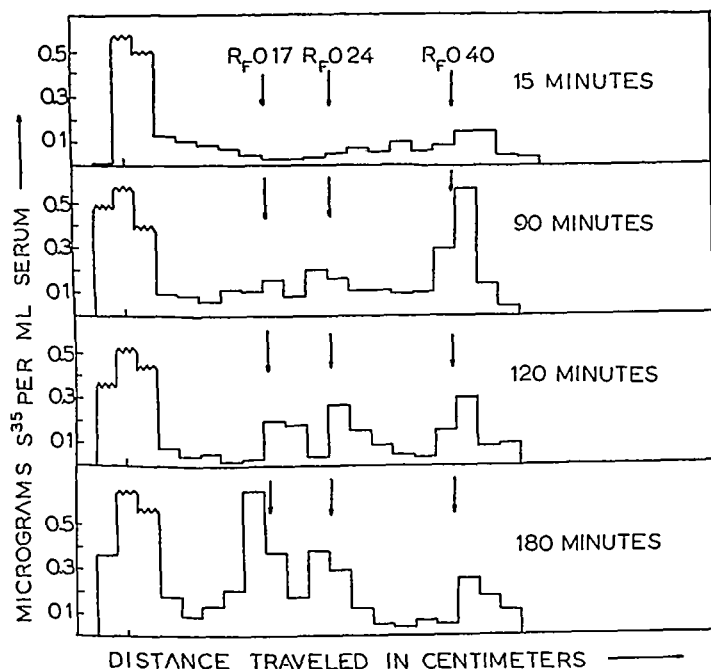


Fig 4 The appearance of radioactive metabolites in serum after intraperitoneal administration of 2.3 mg of cystamine- $S^{35}$  to a mouse weighing 28 gm. Paper chromatography was carried out in isopropanol-ethanol-water at pH 7.4.  $R_F$  0.17 = taurine,  $R_F$  0.24 = hypotaurine, and  $R_F$  0.40 = cystamine

and taurine did not appear until 90 to 120 minutes after the injection, demonstrating that in mice the conversion of cystamine and cysteamine to hypotaurine and to taurine is a fairly slow process, as previously has been found to be the case in rats (13). As will be discussed later, the radioactivity on the starting line represents mainly protein-bound radioactivity.

*Cysteamine Experiments*—Two separate cysteamine experiments which gave nearly identical results were carried out.

A male mouse, weighing 26 gm, was injected intraperitoneally with 16

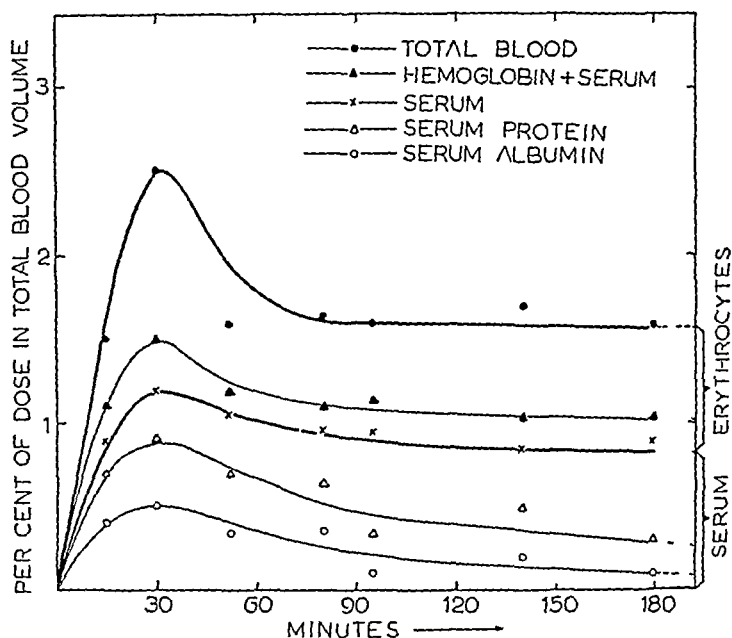


FIG 5 The  $S^{35}$  distribution among certain blood fractions at various times after intraperitoneal administration of 16 mg of cysteamine- $S^{35}$  to a mouse weighing 26 gm

mg of cysteamine- $S^{35}$  hydrochloride. Blood samples were collected at intervals after the injection.

The distribution of the radioactivity among the various blood fractions (Fig 5) was essentially the same as that in the cystamine experiment. Thus, after cysteamine administration as well, an extensive fixation of  $S^{35}$  to intra- and extracellular proteins occurs. The amount of non-protein bound radioactivity in serum was smaller than that in the cystamine experiment.

*Detailed Blood Radioactivity Distribution 30 Minutes after Cystamine Injection*—A male mouse, weighing 24 gm, was injected intraperitoneally with 40 mg of cystamine- $S^{35}$  dihydrochloride. After 30 minutes, the blood was collected by heart puncture. Serum and red cell hemolysate were analyzed in quadruplicate. In Figs 6, A and 7, A typical example.

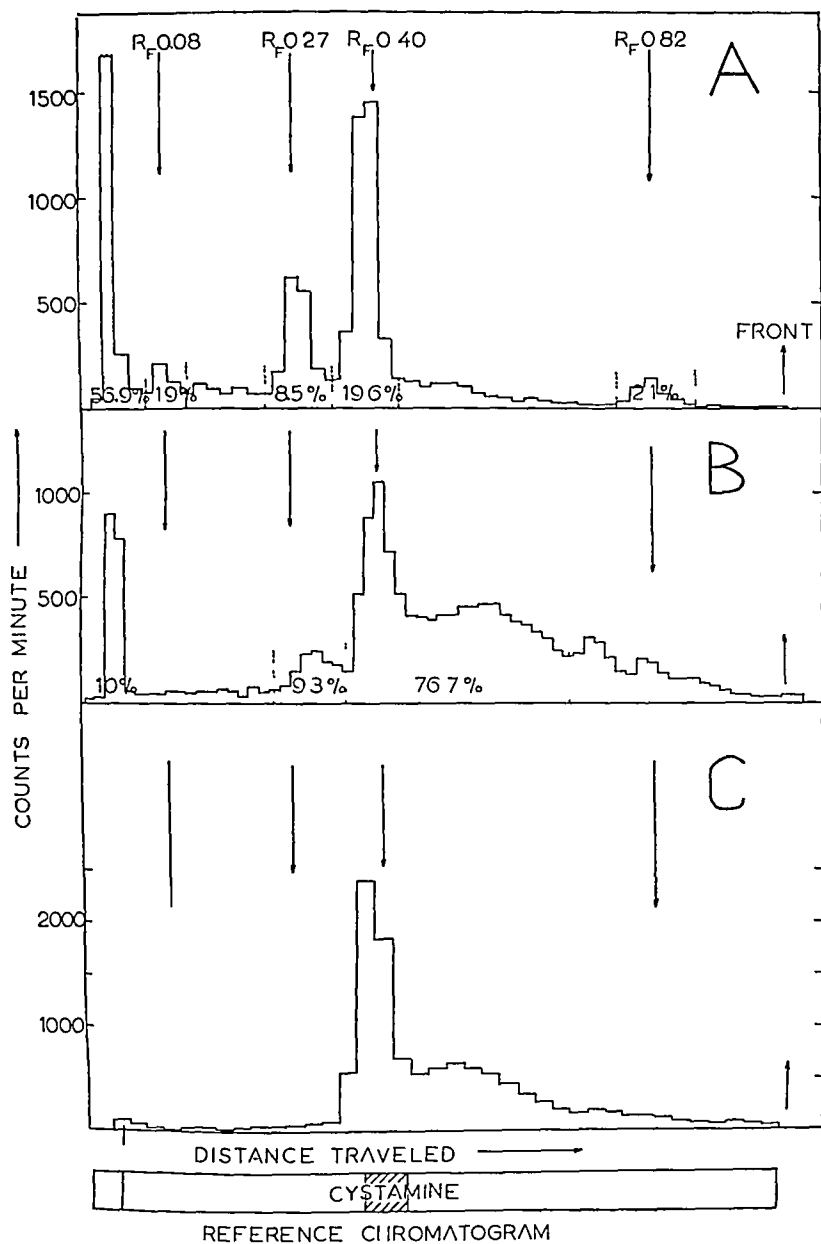


Fig 6 Paper chromatographic separation of the labeled compounds in serum 30 minutes after intraperitoneal administration of 4.0 mg of cysteamine- $S^{35}$  to a mouse weighing 24 gm. A, untreated serum, B, after addition of 5 mg of unlabeled cysteamine per ml of serum, C, control chromatogram of a mixture of 0.7 mg of cysteamine  $S^{35}$  and 4.35 mg of unlabeled cysteamine per ml (phosphate buffer 0.067 M, pH 7.4)

of the radioactivity patterns after paper chromatography are shown. Fig 8 presents the average results.

In agreement with the previous results approximately 50 per cent of the

serum radioactivity occurs in combination with the serum proteins. Approximately 20 per cent of the serum radioactivity appears to be present as free cysteamine or cystamine and 9 per cent as hypotaurine. Two additional constituents appeared on the chromatograms from serum. The compound with  $R_F$  0.08 most probably is identical with the mixed disulfide

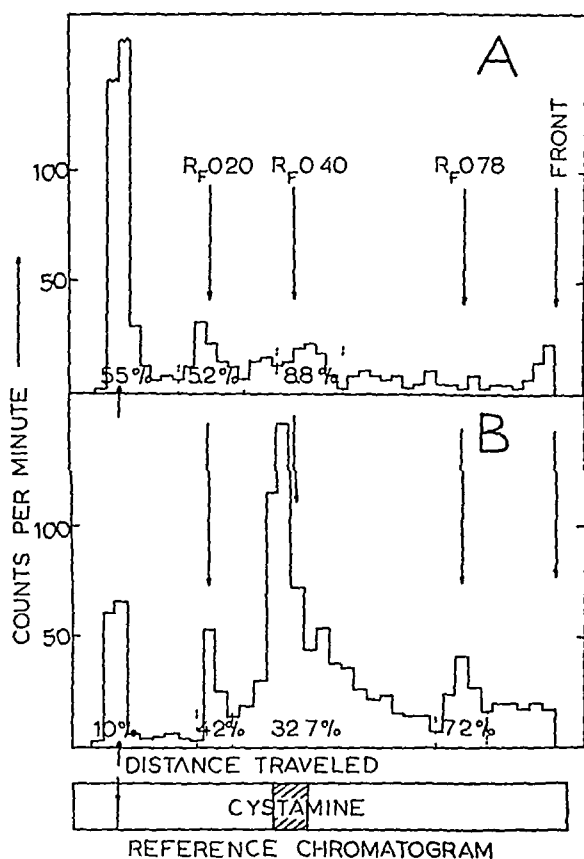


FIG. 7. Paper chromatographic separation of the labeled compounds in hemolysate of red cells collected 30 minutes after intraperitoneal administration of 4.0 mg of cystamine- $S^{35}$  to a mouse weighing 24 gm. A, untreated hemolysate, B, after addition of 2.5 mg of unlabeled cysteamine per ml of hemolysate.

between glutathione and cysteamine, since the  $R_F$  values of the unknown compound and that of the synthetically prepared mixed disulfide are identical in the above system as well as in a phenol system<sup>2</sup>. The compound having an  $R_F$  value of 0.82 has not yet been identified. On the starting line of the serum chromatogram ( $R_F$  0.0) 57 per cent of the radioactivity

<sup>2</sup> Phenol saturated with an aqueous solution of 6.3 per cent trisodium citrate and 3.7 per cent sodium dihydrophosphate. The paper was pretreated with 1 M KCl. The  $R_F$  of the mixed disulfide of glutathione and cysteamine was 0.48 in this system.

was found This is 10 per cent in excess of the amount found by paper electrophoresis to be fixed to the proteins This excess may, at least in part, be present in the form of sulfate (13) The serum radioactivity not accounted for, denoted in Fig 8 by a question mark, represents the sum of the small amounts of radioactivity found between the above well defined peaks

The radioactivity bound to the hemoglobin corresponded to 26 per cent of the total radioactivity of the red corpuscles In the paper chromatograms of the red cell hemolysate 55 per cent of the radioactivity remained

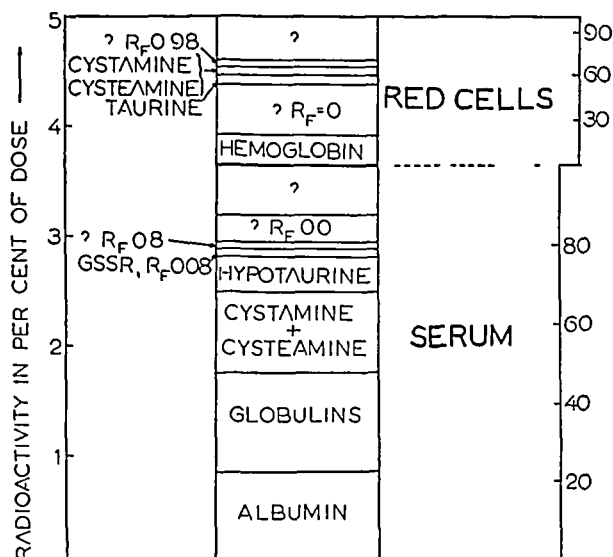


Fig 8 Diagram of the  $S^{35}$  distribution among blood constituents 30 minutes after intraperitoneal administration of 40 mg of cystamine- $S^{35}$  to a mouse weighing 24 gm The mixed disulfide between glutathione and cysteamine is denoted as GSSR

on the starting line Thus, in addition to the hemoglobin-bound radioactivity, 29 per cent of the red cell radioactivity does not migrate in this system It is considered unlikely that this amount could be due to sulfate alone At the  $R_F$  values of hypotaurine and cystamine only small amounts of radioactivity (6 and 6.6 per cent, respectively) could be detected Furthermore, a small amount of radioactivity appeared at the front of the chromatograms

*Chemical Nature of Protein-Bound Radioactivity*—Experiments in this laboratory have demonstrated that when cystamine or cysteamine is incubated with isolated proteins, cysteamine residues become bound to the protein SH groups with the formation of mixed disulfides (7) In order to establish whether the protein-bound radioactivity in the present experi-

ments *in vivo* likewise arose from a fixation of cysteamine residues, an excess of inactive cysteamine was added to the serum and to the red cell hemolysate, and paper electrophoresis and paper chromatography were subsequently carried out

The radioactivity patterns obtained were altered in the expected manner. Thus, the electrophoretograms revealed that the treatment reduced the radioactivity of the proteins by 95 per cent. Also, the amounts of radioactivity remaining on the starting line of the chromatograms (Fig 6, B) were strongly reduced, the radioactivity reappearing at  $R_F$  0.40, the  $R_F$  of cystamine, and in a broad peak with  $R_F$  0.53. An almost identical radioactivity pattern was obtained when, in control experiments, radioactive cystamine was chromatographed after the addition of inactive cysteamine (Fig 6, C). Rechromatography of the radioactivity eluted from a small band at  $R_F$  0.53 (Fig 6, B) established the nature of this peak, since the radioactivity appeared as a single spot at the  $R_F$  of cystamine.

The cysteamine treatment of the red cell hemolysate led to results similar to those described for serum. From a comparison of Fig 7, A with Fig 7, B there can be no doubt that the bulk of the radioactivity released from the starting line by the cysteamine incubation appeared as cystamine.

#### DISCUSSION

The data presented in this paper demonstrate that, upon administration of cystamine or cysteamine to mice, the major part of cystamine or cysteamine in blood occurs in chemical combination with the blood proteins and with simpler compounds such as glutathione. We have previously shown (7) that, *in vitro*, cystamine and cysteamine may form significant amounts of mixed disulfides with the free SH groups of proteins and simpler compounds. The importance of mixed disulfide formation is also evident from the recent study of Kolthoff *et al* (15) who have measured the equilibrium constants of several SH-SS systems. Previously, mixed disulfides were believed to be unstable intermediates, occurring only in insignificant amounts in exchange reactions between thiols and disulfides (16). The present data, which constitute strong evidence that mixed disulfides are formed to a large extent *in vivo*, suggest that the phenomenon is of general biochemical significance.

The finding that, after protective doses of labeled cystamine or cysteamine to mice, only a minor fraction of the blood radioactivity is present as free cystamine or cysteamine supports our conclusion from previous studies *in vitro* (12, 7) that inactivation of radicals by cystamine or cysteamine randomly distributed throughout the water phase of the tissues cannot adequately explain the protective ability of these compounds *in*

*in vivo* The slow rate of formation of taurine and hypotaurine rules out the possibility that they can be responsible for the x-ray protection afforded by cystamine or cysteamine

The results focus the interest on the interaction of cystamine and cysteamine with biological target groups. It is of interest to note that the protein fixation is maximal during the initial 30 minutes after the administration of the dose, *i.e.* during the period when optimal protection against ionizing radiation is found. It is also significant that essentially the same extent of protein binding was obtained whether cystamine or cysteamine was administered. It will be recalled that these two compounds have been found to have nearly identical protective ability (17, 18). The data suggest that the extensive, temporary binding of cystamine and cysteamine to body constituents is causally related to the ability of these compounds to protect against ionizing radiation.

#### SUMMARY

The metabolism in mice of the x-ray protective agents cystamine and cysteamine has been reinvestigated with the aid of  $S^{35}$ -labeled compounds of extremely high specific activity.

In principle the same results were obtained whether cystamine or cysteamine was administered. During the period when these compounds offer optimal protection, the bulk of the cystamine and cysteamine in blood occurred bound to intra- and extracellular proteins and to other blood constituents. Evidence is presented that this is due to the formation of mixed disulfides between cysteamine and the SH groups of the body constituents.

The radiobiological significance of the data has been discussed.

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# THE SYNTHESIS OF S<sup>35</sup>-LABELED HYPOTAURINE AND ITS METABOLISM IN RATS AND MICE\*

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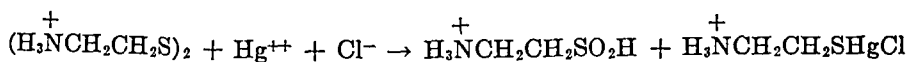
(Received for publication, April 10, 1956)

Hypotaurine (2-aminoethanesulfinic acid) is known to be formed from cysteine in rats (1). In mammals this conversion may proceed either by way of cysteinesulfinic acid (2), cystamine-cysteamine (3), or possibly by way of cystamine disulfoxide (4, 5). However, the study of the metabolism of hypotaurine has been hampered by the difficulties involved in preparing the pure compound (6-8). The only study known to the authors in which hypotaurine was used is the demonstration by Cavallini *et al.* (9) that the administration of a relatively large dose of hypotaurine to a rat led to an increase in the urinary excretion of taurine.

In the present paper a simple procedure for the synthesis of hypotaurine from cystamine is reported. The method has enabled us to prepare S<sup>35</sup>-labeled hypotaurine with a specific activity of 4 mc of S<sup>35</sup> per mg of sulfur. With the labeled hypotaurine its conversion to taurine in mice and rats was confirmed. In addition, an extensive and rapid sulfate formation was demonstrated.

## EXPERIMENTAL

*Synthesis of Hypotaurine*—1.05 gm of HgSO<sub>4</sub>, dissolved in 7 ml of 2.25 N H<sub>2</sub>SO<sub>4</sub>, were added, at room temperature, to a stirred solution of 100 mg of cystamine dihydrochloride in 3 ml of 2.25 N H<sub>2</sub>SO<sub>4</sub>. This treatment dismutates cystamine according to the following reaction:



The solution was made 15 per cent with respect to ethanol and the precipitated mercaptide was removed by centrifugation. The supernatant fluid was reduced to a small volume by lyophilization and again clarified by centrifugation.

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† Fellow of the Norwegian Cancer Society.

The hypotaaurine was isolated by cation exchange chromatography on a Dowex 50 column. The column,  $10 \times 0.6$  cm, was pretreated with 2 N NaOH, 1 N HCl, and 0.2 N  $NH_3$  in succession. The hypotaaurine was eluted with 0.2 N  $NH_3$  and appeared in a pure state in a 2 to 3 ml range when 50 to 100 ml of effluent had been collected. The yield at this stage was 14 to 23 per cent (theoretical yield 25 per cent). Upon paper chromatography in an ethanol-isopropanol system (10), all the radioactivity appeared in a narrow band at  $R_F$  0.25, coinciding with that of synthetic hypotaaurine, kindly furnished by Dr J. Awapara. Crystalline hypotaaurine (m.p.  $170^\circ$ ) was obtained after freeze-drying of the eluate and crystallization from water-ethanol-ether mixtures.

$S^{35}$ -labeled hypotaaurine was similarly prepared from 10 mg of  $S^{35}$  cystamine dihydrochloride (11). The yield was 2 mg. The specific activity was identical with that of the cystamine (4 mc of  $S^{35}$  per mg of S).

*Animal Experiments*—A male mouse weighing 28 gm was injected intraperitoneally with 1.26 mg of hypotaaurine- $S^{35}$ . At intervals blood samples were taken from the tail, and the serum proteins and the hemoglobin were isolated by paper electrophoresis. Aliquots of the serum samples collected after 15 and 30 minutes were analyzed in duplicate by descending paper chromatography in the ethanol-isopropanol system. The radioactivity on the chromatograms and on the electrophoretograms was measured by means of a strip counter. The details of the analytical procedures have been described previously (12).

The mouse was placed in a glass jar and urine was collected during the first and second 24 hour period. The free sulfate of the urine samples was precipitated as barium sulfate and the radioactivity was determined after being plated at saturation thickness. Aliquots from the 24 hour sample, as well as from the freshly voided urine samples obtained 4 and 24 hours after the injection, respectively, were analyzed in duplicate in the above chromatographic system.

Two experiments were carried out with rats. Since the results were almost identical, only one of the experiments will be described in detail. A male rat weighing 225 gm was injected with 0.5 mg of hypotaaurine- $S^{35}$ . The animal was placed in a metabolism cage and urine was collected for 6 days. The free sulfate of the urine samples was precipitated with benzidine at pH 5, recrystallized, and finally precipitated as barium sulfate. The total sulfate was obtained as barium sulfate after acid hydrolysis for 20 minutes. The total sulfur was precipitated after wet oxidation (3).

The validity of the procedures described for the determination of free and total sulfate radioactivity was checked by the following experiment. 0.1 mg of hypotaaurine- $S^{35}$  was added to a 10 ml portion of urine, and free and total sulfate were isolated as described above. No more than 2.74 and

6.24 per cent of the added hypotaurnine radioactivity were recovered in the free sulfate and in the total sulfate fraction, respectively

### RESULTS AND DISCUSSION

In the mouse experiment, the paper chromatograms from serum (Fig 1) revealed that, 30 minutes after the injection, the amount of taurine ( $R_F$  0.13) already exceeded that of labeled hypotaurnine ( $R_F$  0.23), demonstrat-

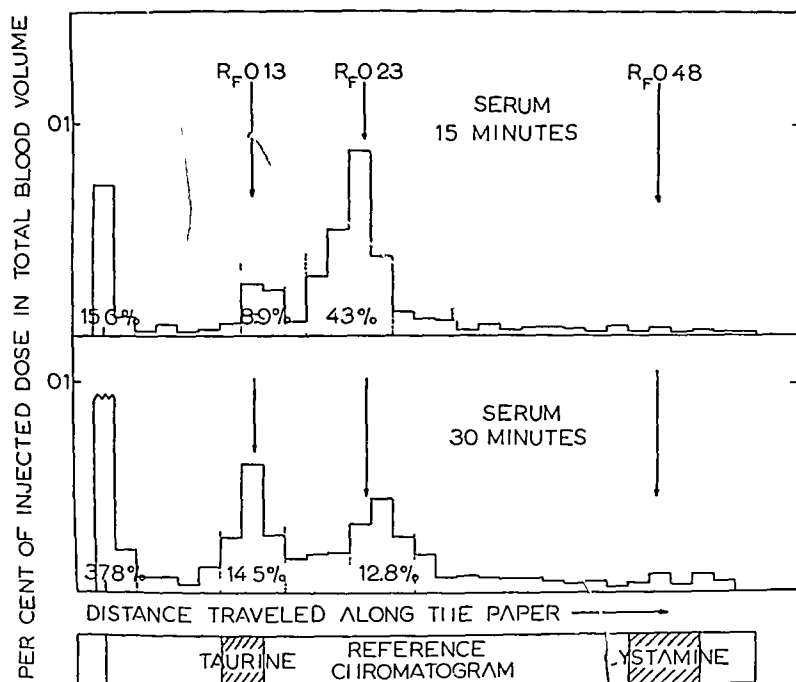


Fig 1 Paper chromatograms of serum samples after intraperitoneal administration of 1.26 mg of hypotaurnine- $S^{35}$  to a mouse weighing 28 g. In the system used the  $R_F$  of hypotaurnine is 0.25 and that of inorganic sulfate 0.0. For further details see the text

ing that this conversion, in contrast to previous statements (3, 13), takes place at a fairly rapid rate. The most striking fact apparent from the chromatograms is the presence of a large fraction of the radioactivity on the starting line. In the light of the urine findings to be discussed below, this radioactivity no doubt must be attributed to the presence of inorganic sulfate, which fails to migrate in this system. The serum proteins likewise fail to migrate in this system, but the paper electrophoretograms showed that no radioactivity was bound to the proteins.

In Fig 2 the results of the chemical and chromatographic analyses of the pooled urine samples are shown. It is apparent that approximately

50 per cent of the urine radioactivity was recovered as sulfate, partly in free and partly in esterified form. The total activity as sulfate agreed closely with the activity retained on the starting line of the paper chromatograms.

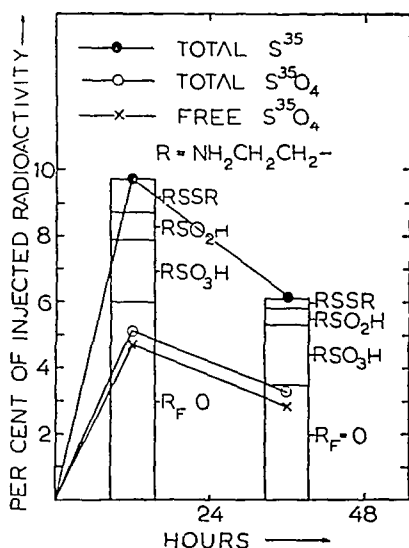


FIG 2

FIG 2 Radioactivity distribution in urine following the intraperitoneal administration of 1.26 mg of hypotaurine- $S^{35}$  to a mouse weighing 28 gm. Analyses of the pooled 0 to 24 and 24 to 48 hour samples are shown. The columns represent the results of paper chromatographic analyses.

FIG 3 Paper chromatographic separation of the labeled compounds in freshly voided urine samples collected by capillary 4 and 24 hours after intraperitoneal administration of 1.26 mg of hypotaurine- $S^{35}$  to a mouse weighing 28 gm. For further details see the text.

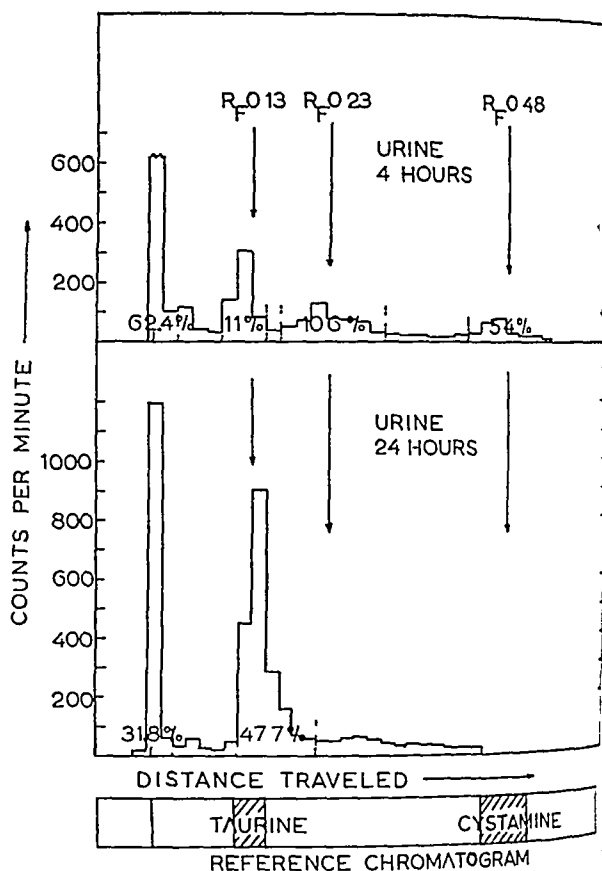


FIG 3

The paper chromatograms showed that during the first 24 hour period approximately equal amounts of taurine and hypotaurine were excreted, whereas during the subsequent 24 hour period predominantly taurine was excreted.

The chromatograms of the freshly voided urine samples obtained 4 and 24 hours after the injection of hypotaurine (Fig 3) verified the extensive

and rapid sulfate formation and also the hypotaurine to taurine relationship mentioned above. In addition, a constituent with the  $R_F$  of cystamine was present in the urine collected after 4 hours. This constituent was absent in the serum chromatograms and in the urine collected after 24 hours. Since this peak was only found in urine samples in which substantial amounts of hypotaurine were present (Figs 2 and 3), it appears plausible that in urine a spontaneous reduction of hypotaurine may take place with the formation of cystamine and the intermediary reduction steps (cystamine sulfoxides). This view is supported by our finding that hypotaurine, in buffered solutions of pH 5 to 7, is slowly converted to cystamine when SH compounds are present. In the light of this evidence, it appears likely that the cystamine disulfoxide, isolated by Cavallini *et al* from the urine of rats fed cystine (5), represents a urinary reaction product rather than an intermediate in the hypotaurine formation.

TABLE I  
*Urinary Excretion of  $S^{35}$  Following Intraperitoneal Administration of  
0.5 Mg of Hypotaurine- $S^{35}$  to Male Rat Weighing 225 Gm*

	Per cent of administered radioactivity				
	1st day	2nd day	3rd + 4th days	5th + 6th days	Total recovered
Free $SO_4$	4.7	1.1	2.3	1.9	10.0
Total "	5.5	1.3	3.2	2.0	12.0
" S	12.8	2.9	8.6	4.2	28.5

In the rat experiment, in which a quantitative collection of urine was achieved, about 30 per cent of the injected radioactivity could be accounted for in the urine (Table I). Also in this case almost 50 per cent of the radioactivity in the urine was recovered as sulfate. It is clear from the check experiment mentioned above that only a negligible fraction of this radioactivity could be due to coprecipitated hypotaurine.

Although in the mouse experiment no more than 16 per cent and in the rat experiment no more than 30 per cent of the injected radioactivity was accounted for, the data demonstrate beyond doubt that, in addition to the conversion of hypotaurine to taurine, an extensive sulfate formation takes place. This preferential conversion of hypotaurine to sulfate raises the possibility that a significant fraction of the sulfate formation from cysteine proceeds by way of hypotaurine. Since attempts to demonstrate a direct liberation of sulfur from cystamine and cysteamine have consistently failed and, furthermore, since there is only a limited formation of sulfate from taurine (3), hypotaurine appears to be an obligatory intermediate in the sulfate formation from cystamine and cysteamine (14). Our data also in-

icate that hypotaurine cannot be reduced biologically, as no cystamine peak could be detected in the chromatograms from serum

#### SUMMARY

A simple procedure for the synthesis of hypotaurine (2-aminoethanesulfonic acid) is reported. By this method hypotaurine-S<sup>35</sup> has been prepared and its metabolism has been studied in rats and mice.

The conversion of hypotaurine to taurine is confirmed. Furthermore, a rapid and extensive formation of sulfate from hypotaurine is demonstrated.

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# CONCENTRATION AND COMPOSITION OF SERUM LIPOPROTEINS OF CHOLESTEROL-FED AND STILBESTROL-INJECTED BIRDS\*

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Most of the studies on serum lipoproteins have dealt with the concentrations of specific groups of these lipoproteins under a variety of experimental and clinical conditions. Recently we presented a method for measuring the lipide and protein compositions of four lipoprotein fractions that accounted for total serum lipoproteins (1). The separation of the fractions was based on the ultracentrifugal flotation technique as developed by Lindgren *et al* (2), and each fraction was analyzed for protein, phospholipides, free and esterified cholesterol, and triglycerides. Although considerable variation in the concentrations of the various lipoprotein fractions was observed among the five species studied, namely, man, dog, rabbit, rat, and chicken, the composition of each lipoprotein fraction with respect to protein and various lipides was similar for the sera of these five species.

The present report deals with the concentrations and the composition of the various lipoprotein fractions in the serum of birds that were either fed cholesterol-rich diets or injected with stilbestrol. The interest in these two experimental conditions lies in the fact that both induce arteriosclerosis in the bird and that the latter induced arterial degeneration even when the birds were fed diets low in lipide content (3, 4).

## EXPERIMENTAL

*Treatment of Animals*—2 month-old white Leghorn cockerels were divided into three groups. The first group, which served as controls, was fed Purina broiler chow *ad libitum*, the second group was fed *ad libitum* a diet consisting of Purina broiler chow to which had been added 1 per cent cholesterol and 5 per cent Wesson oil, and the third group received, at bimonthly intervals, subcutaneous implants of 24 mg of diethylstilbestrol and were fed Purina broiler chow *ad libitum*.

*Nomenclature of Lipoprotein Fractions*—In the interest of clarity and in order that the lipoproteins analyzed in this investigation may be compared

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with those isolated and characterized by other means, both the nomenclature used in this paper and those of other investigators are presented in Table I. From the close correspondence in composition between our Fraction C and  $\alpha_1$ -lipoprotein, as isolated by the Cohn system and first analyzed by Oncley and Gurd (8), we have concluded that the two are identical. Complete analyses for  $\alpha_2$ -lipoprotein have not been published, but its identity with our Fraction B seems to be indicated from the chole-

TABLE I  
*Nomenclature of Lipoprotein Fractions*

Density range used for separation	Designation of fractions used here	Gofman's classification	Electrophoresis classification
$D^{1.006*}$	A1†	S <sub>f</sub> 20 and higher†	No corresponding fraction
$D^{1.063}_{1.006}$	A2†	S <sub>f</sub> 0-20†	$\beta$ -Lipoprotein (5, 6)
$D^{1.107}_{1.063}$	B	1.075§	$\alpha_2$ -Lipoprotein (6)
$D^{1.220}_{1.107}$	C	1.145§	$\alpha_1$ -Lipoprotein (8)
$D_{1.220}$	D		

\* The superscript represents the highest density medium used for separation of the fraction, and all lipoproteins in this fraction will have densities less than the value of the superscript, i.e., these lipoproteins will undergo flotation when centrifuged in a medium of density represented by the superscript. The subscript represents the lowest density medium used in preparing the fraction, and all lipoproteins in this fraction will have densities greater than the value of the subscript, i.e., the lipoproteins will undergo sedimentation when centrifuged in a medium of density represented by the subscript. The media are prepared at 20°.

† In our previous paper (1) we used the term Fraction A to designate the combined Fractions A1 and A2, i.e., Fraction  $D^{1.063}$ .

‡ These are the low density lipoproteins (2). The chylomicrons are most likely lipoproteins of S<sub>f</sub> 400 and higher.

§ The figures represent their approximate hydrated densities. These are the high density lipoproteins (7).

sterol to phospholipide ratios (1). Neither the  $\beta$ -lipoprotein nor our Fraction A2 is homogeneous, although the compositions of both fractions are similar (1).

### *Lipoprotein analyses*

*Preparation of Lipoprotein Fractions*—The method used here is a modification of that described earlier (1), and only the pertinent changes are given. In normal birds, good separation of the lipoprotein fractions was obtained when 5 ml of a serum were centrifuged in a single tube. In the experiments with cholesterol-fed and stilbestrol-injected birds it was found necessary to centrifuge several smaller samples of serum (0.5 to 2 ml)

to obtain a clear separation of the low density lipoproteins<sup>1</sup> The serum sample was transferred to a rotor tube, and enough 11 per cent NaCl was added to yield a final volume of 9 ml with a density approximately that of serum (1.006) The tube (No I in Fig 1) was capped, and the contents were mixed by inversion Tube I was then centrifuged for 24 hours at 30,000 r p m After centrifugation, Fraction A1, which was at the top of the

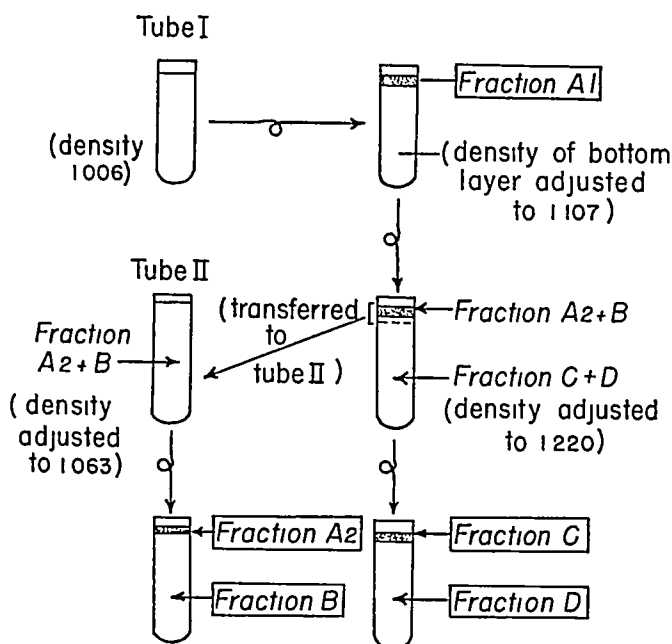


FIG 1 Scheme for lipoprotein fractionation  $\rightarrow$  represents centrifugation in the preparative ultracentrifuge as described in the text Only the fractions enclosed in the rectangles were removed for analysis

tube, was transferred to a 50 ml centrifuge tube with a short, conical bottom by means of the suction device described previously (1) The density of the solution remaining in Tube I was adjusted to 1.107 by the addition of 1.35 gm of solid KBr, and enough 11 per cent NaCl was added to bring the volume up to 9 ml Tube I was capped, and the contents were mixed by inversion until the solid KBr dissolved This tube was then centrifuged for 20 hours at 30,000 r p m

After centrifugation, the top layer contained Fractions A2 and B, and the bottom layer contained Fractions C and D The top layer in Tube I was transferred to another rotor tube (No II in Fig 1) calibrated at 4 ml

<sup>1</sup> In cholesterol-fed birds the presence of a lipemia was associated with an exaggerated tendency to gelation of the sera, whereas in the stilbestrol-treated birds the tendency to gelation was less than that in normal birds Frequently the gelation in the cholesterol-fed birds was so great that the sera were not suitable for analysis

The contents of Tube II were brought to a volume of 4 ml with 14 per cent KBr. The density of this mixture was adjusted to 1.063 by the addition of 5 ml of 5 per cent KBr. Tube II was capped, and the contents were mixed by inversion.

Next, 1.5 gm of solid KBr were added to the residue in Tube I, the tube was shaken briefly, and the volume of the mixture was made to 9 ml by addition of a 14 per cent KBr solution. Tube I was capped, and the contents were mixed by inversion until the KBr dissolved. The density of this solution was 1.220 at 20°. Tubes I and II were centrifuged at 30,000 r p m for 24 hours.

This third centrifugation separated the contents of Tube II into a narrow yellow layer (Fraction A2) at the top and a clear one (Fraction B) at the bottom. Tube I contained a dark yellow layer at the top (Fraction C) and a clear lower layer (Fraction D).

Fractions A2 and C were transferred to individual 50 ml centrifuge tubes. Fraction B was divided into two parts, and each part was transferred to a separate 50 ml centrifuge tube. Fraction D was transferred to a 125 ml Erlenmeyer flask.

*Extraction and Purification of Lipides*—The lipides were extracted from each fraction with a 6:2:1 mixture of alcohol, ether, and chloroform as described previously (1). The KBr in the lipide extracts interferes with the determination of cholesterol and esterified fatty acids, and it was therefore removed by the following procedure. The combined extracts were transferred to 125 ml Erlenmeyer flasks provided with closed side arms of 2 ml capacity. The extracts were evaporated, on a steam bath, to a small volume. The residual solvent consisted mainly of water, and to it enough 95 per cent ethanol was added to yield an ethanol concentration of 50 per cent. About 20 ml of a petroleum ether solution (b.p. 30–70°), containing 5 per cent chloroform, were added to each flask. The flasks were heated on a steam bath until the volume was reduced about one-half. The mixture was kept at room temperature until bubbling stopped, and the petroleum ether was then decanted into a volumetric flask. The alcohol-water phase was trapped in the side arm during decantation. The petroleum ether extraction was repeated three more times. The extract contained negligible amounts of salt.

*Determination of Protein and Lipides*—Protein was determined by the method of Gornall *et al.* (9). Phospholipide was determined by a combination of the methods of King and Horecker *et al.* (10, 11). Free and total cholesterol were precipitated as the digitonide and determined by the colorimetric method of Zlatkis *et al.* (12), in which an iron reagent is employed. Esterified fatty acids contained in phospholipides and triglycerides

erides were determined by the method of Stern and Shapiro (13)<sup>2</sup> The amounts of fatty acid esterified with cholesterol were calculated from values obtained for free and total cholesterol

### Results

*Concentration of Total Serum Lipoprotein and of Its Lipide and Protein Components*—Cholesterol feeding resulted in a 4-fold increase in the concentration of total serum lipoproteins. In the stilbestrol-injected birds, the increases were much greater, *ie* in the neighborhood of 15- to 25-fold (Table II). Both lipide and protein components participated in these increases. The increase in the cholesterol moiety was about the same in

TABLE II

*Concentration of Total Serum Lipoprotein and of Its Lipide and Protein Components in Normal, Cholesterol-Fed, and Stilbestrol-Injected Birds*

All values are expressed as mg per 100 ml of serum. These values are the sums of the values obtained for Fractions A1, A2, B, and C given in Table III.

Constituent	Normal	Cholesterol fed*	Stilbestrol-injected†		
			Bird 1	Bird 2	Bird 3
Total lipoprotein	928	3938	13,033	19,021	24,010
Lipide moiety of lipoprotein					
Total cholesterol	103	997	631	986	1,386
Phospholipide	217	586	2,381	3,858	4,414
Triglyceride	187	1246	8,080	11,609	14,792
Protein moiety of lipoprotein	374	626	1,738	2,351	2,899

\* These birds were sacrificed 2 months after the start of the cholesterol feeding.

† These birds were sacrificed 6 months after the first injection of stilbestrol.

both types of birds, but for all the other components, namely, phospholipide, triglycerides, and protein, the response was much more pronounced in the estrogen-treated birds than in the cholesterol-fed birds.

*Concentration and Composition (in Terms of Mg Per 100 Ml Serum) of Five Serum Lipoprotein Fractions*—The most pronounced changes in the cholesterol-fed and the stilbestrol-injected birds were observed in Fractions A1 and A2 (Table III), and the rise in the concentration of these two fractions accounts for practically all of the increases in the total lipoprotein levels. The responses of Fractions A1 and A2 were not the same, that of Fraction A1 by far exceeded that of Fraction A2. Virtually no difference

<sup>2</sup> In our hands, long chain fatty acid esters of cholesterol fail to form hydroxamic acids under conditions described by Stern and Shapiro (13). Their method is satisfactory for the determination of phospholipide fatty acids and triglyceride fatty acids.

TABLE III

*Concentration and Composition of Five Individual Lipoprotein Fractions in Sera of Normal, Cholesterol-Fed, and Stilbestrol-Injected Birds*

Individual values are recorded for the stilbestrol-injected birds. In the case of the normal (three birds) and cholesterol-fed birds (three birds), the agreement found in the various values was good, and only average values are recorded

Lipoprotein fraction	Condition of bird	Mg of fraction per 100 ml serum	Composition of fraction				
			Protein	Phospholipide	Free cholesterol	Esterified cholesterol	Triglycerides
			Mg found in lipoprotein fraction per 100 ml serum				
$D_{1006}^1$ or A1	Normal	*	*	*	*	*	*
	Cholesterol-fed	2,514	208	309	157	858	9,000
	Stilbestrol-injected, Bird 1	11,941	1435	2160	302	454	7,000
	" " 2	17,344	1962	3402	590	490	10,000
	" " 3	22,163	2460	4030	563	1210	13,000
$D_{1006}^{1063}$ or A2	Normal	225	35	43	15	41	91
	Cholesterol-fed	815	112	149	91	252	211
	Stilbestrol-injected, Bird 1	891	189	190	23	45	444
	" " 2	1,457	267	415	65	41	663
	" " 3	1,634	325	345	44	78	833
$D_{1063}^{1107}$ or B	Normal	64	25	17	3	8	11
	Cholesterol-fed	79	34	10	4	17	14
	Stilbestrol-injected, Bird 1	38	19	2	1	1	10
	" " 2	102	37	23	4	5	33
	" " 3	75	38	6	2	2	21
$D_{1107}^{1220}$ or C	Normal	639	314	157	15	68	80
	Cholesterol-fed	530	272	118	15	86	39
	Stilbestrol-injected, Bird 1	163	95	29	1	7	31
	" " 2	118	85	18	3	5	1
	" " 3	138	76	24	2	4	37
$D_{1220}$ or D	Normal	†	†	22	1†	†	†
	Cholesterol-fed	†	†	26	2	7	†
	Stilbestrol-injected, Bird 1	†	†	210	8	2	150
	" " 2	†	†	261	10	5	151
	" " 3	†	†	285	13	5	100

\* The concentrations were too low to measure

† These values cannot be reported since Fraction D contained serum albumin and globulins in addition to lipoproteins

‡ The concentrations were too low to measure. The value in the free cholesterol column for the normal cockerel is actually that for total cholesterol

was observed between the two types of treated birds in the amounts of Fraction A2, but Fraction A1 increased far more in the stilbestrol-injected than in the cholesterol-fed birds

Fraction B failed to respond to either of the treatments

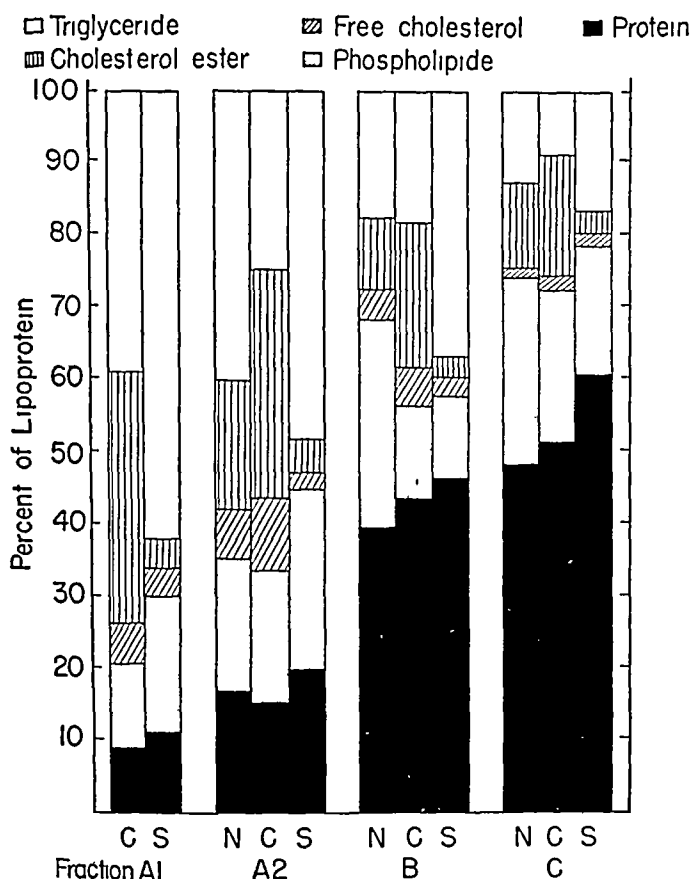


FIG 2 The percentage composition of four lipoprotein fractions in normal, cholesterol-fed, and stilbestrol-injected birds. N = normal bird serum, C = cholesterol-fed bird serum, S = stilbestrol-injected bird serum

Interestingly enough, stilbestrol injections reduced the levels of Fraction C, whereas cholesterol feeding had no effect

No response of Fraction D to cholesterol feeding occurred, but a definite rise in this fraction followed the estrogen injections

*Changes Induced in Percentage Composition of Four Lipoprotein Fractions by Cholesterol Feeding and Stilbestrol Injection*—The percentages of protein in Fractions A1 and A2 were unaffected by cholesterol feeding and stilbestrol treatment, a small increase did occur in Fractions B and C (Fig 2). The principal changes that resulted from the administrations of cholesterol

and stilbestrol were observed in cholesterol esters and triglycerides. It is of interest that the feeding of cholesterol increased the percentage of cholesterol esters in each of the four fractions (A1, A2, B, and C), whereas the stilbestrol injections reduced the percentages of this lipid constituent to a considerable extent in each fraction. The percentage of triglycerides in all four fractions was decreased or unaffected after cholesterol feeding, but was increased by the stilbestrol treatments.

The *relative* distribution of free and esterified cholesterol in Fractions A1, A2, B, and C in the cholesterol-fed birds was the same as that observed in the normal birds, *i.e.*, the percentage of esterified cholesterol was much higher than that of free cholesterol (Fig. 2). When stilbestrol was injected, the percentages of free and esterified cholesterol in each lipoprotein fraction were about the same.

#### DISCUSSION

The results of the present study demonstrate that prolonged cholesterol feeding and stilbestrol injections bring about changes not only in the level of serum lipoproteins, which was to be expected, *but also in the lipid composition* of our four lipoprotein fractions. After cholesterol feeding, only Fractions A1 and A2 increased in concentration, a finding in agreement with the observations of other investigators (14, 15). Stilbestrol administration also augmented the levels of Fractions A1 and A2, but it is of interest that this treatment was also associated with a reduction in the concentration of Fraction C in serum.

Despite the fact that both cholesterol feeding and stilbestrol administration induced arteriosclerosis in birds, the changes they brought about in the composition of the serum lipoprotein fractions differed widely. Thus, in the birds fed cholesterol, the percentages of cholesterol esters in Fractions A1, A2, B, and C were increased, but in the stilbestrol-injected birds the percentages of this lipid constituent fell in all four fractions. On the other hand, the percentages of triglycerides in Fractions A1, A2, B, and C were reduced by cholesterol feeding, but increased by stilbestrol injection. Both experimental procedures increased the amounts of protein in the total serum lipoprotein, particularly in Fraction A1, but the protein increases observed with stilbestrol injections were about 10 times those found with cholesterol feeding.

The concentration of Fraction D was elevated in the stilbestrol-injected birds, an observation which raises the question as to the nature of this fraction. Fraction D is a residue in our procedure, and no further separation of lipoproteins from it can be achieved by flotation at densities higher than 1.2 because these higher densities approach those of the non-lipide-containing proteins. We previously assumed that the lipides found in Fraction D

are components of a lipoprotein (1) The following evidence has been obtained on the lipid nature of the phosphorus-containing substances in Fraction D (a) This fraction contains phosphorus which is soluble in a 3:1 alcohol-ether mixture and in a 6:2:1 alcohol-ether-chloroform mixture (1) Similar findings have been reported by Hack (16) and by Havel *et al* (17) (b) When the proteins in Fraction D are precipitated and washed with trichloroacetic acid, the phosphorus in the precipitate can be extracted with a 6:2:1 alcohol-ether-chloroform mixture, and the phosphorus so extracted accounts for all of the original phosphorus (18) Havel *et al* (17) have shown that this phosphorus cannot be removed by dialysis and is precipitated with the proteins by zinc hydroxide Thus this phosphorus is neither inorganic nor the common, acid-soluble organic phosphorus (c) The ratios of cholesterol to phospholipid in Fraction D were constant for each species, and these ratios for Fraction D differed greatly from those observed for Fractions A (Table I), B, and C (1) This would argue against the view that the lipids in Fraction D represent lipids incompletely separated from the other fractions (d) Three fractions, A, B, and C, were labeled in the dog by the injection of inorganic  $P^{32}$  Fractions A, B, and C were isolated, dialyzed, and incubated separately with unlabeled serum obtained from another dog (18) The four fractions were reisolated from the incubation mixture, and the specific activity of the lipid phosphorus was determined for each fraction In each case, Fraction D was labeled and its specific activity was the same as those of the other three fractions These  $P^{32}$  findings show that, whatever the nature of these phosphorus-containing compounds, the phosphorus exchanges readily with that present in the three lipoprotein fractions, A, B, and C

Evidence recently obtained by Hack suggests that the lipid phosphorus in Fraction D may not be present as a typical lipoprotein (16) He stated that 70 per cent of the phosphorus in the residual fraction, which corresponds to our Fraction D, is contained in a compound that he tentatively identified as threonylphosphoserylglutamic acid In this connection it should be noted that, although lipopeptides have been reported to be present in serum (19, 20), it was subsequently shown that the peptides and phospholipids of the lipopeptides can be readily separated by paper chromatography (21) The exact nature of the phosphorus compound in our Fraction D remains to be elucidated

#### SUMMARY

1 Birds were either fed a high cholesterol diet for 2 months or injected with stilbestrol for 6 months Both procedures produce arteriosclerosis The serum lipoproteins were fractionated into five parts by a procedure that allowed us to determine the amounts of each in serum and the lipid and



protein composition of each fraction. These fractions have been designated A1 or  $D_1^{1.006}$ , A2 or  $D_1^{1.063}$ , B or  $D_1^{1.107}$ , C or  $D_1^{1.220}$ , and D or  $D_1^{1.220}$ , where the superscript and the subscript define the density range used in separation of the lipoproteins.

2 Both experimental procedures induced changes in the composition of the lipoprotein fractions as well as in their levels.

3 Cholesterol feeding resulted in an increase in the concentrations of Fractions A1 (S<sub>v</sub> 20 and greater) and A2 (S<sub>v</sub> 0-20) and in an increase in the percentage of cholesterol esters in Fractions A1, A2, B, and C.

4 Stilbestrol injections increased the concentrations of Fractions A1 and A2 and decreased that of Fraction C. The percentages of cholesterol were markedly reduced in Fractions A1, A2, B, and C, whereas the triglyceride contents of these same fractions were increased.

5 The lipides in the highest density Fraction D were increased greatly in the stilbestrol-treated bird. The nature of this fraction is discussed.

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# EFFECT OF ESTRADIOL ON THE METABOLISM OF SERINE-3-C<sup>14</sup> IN SURVIVING UTERINE SEGMENTS\*

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Previous studies on the metabolism of formate and glycine in surviving uterine segments from estrogen-treated rats have revealed the high sensitivity of certain "1-carbon" metabolic pathways as indicators of early estrogenic action (1, 2). In the present paper these studies have been extended with serine-3-C<sup>14</sup>. In response to 6 hours pretreatment with estradiol, the incorporation of radioactivity into the purine bases of mixed nucleic acids was accelerated 5- to 6-fold, while the incorporation into protein or oxidation to carbon dioxide was accelerated to a lesser extent, in contrast, the estrogen had no significant effect on the quantity of radioactivity converted into the lipid fraction. The results obtained with the addition of formate, acetate, and glycine as non-radioactive cosubstrates are described. The possible role of estrogen in these processes is discussed.

## Methods

As described in an earlier publication (1), eighteen ovariectomized rats<sup>1</sup> were injected in the tail vein with 1 ml of a buffered saline containing 10  $\gamma$  of estradiol-17 $\beta$ , eighteen control rats received the buffered saline only. After 6 hours the uterine horns were removed, each horn being cut into four equal segments. The segments were pooled as control and estrogen-treated tissues, respectively. Twelve segments, equivalent to one and one-half uteri, were selected at random and placed into chilled Warburg flasks. Duplicate flasks were run in all cases. The reaction medium consisted of 2 ml of Robinson's medium (3) containing 0.022 M glucose and 0.002 M DL-serine-3-C<sup>14</sup> (1  $\mu$ c per  $\mu$ mole)<sup>2</sup> in the following combinations with non-radioactive cosubstrates: (1) none, (2) 0.005 M sodium formate, (3) 0.005 M glycine, (4) 0.005 M sodium formate plus 0.005 M glycine, (5) 0.005 M sodium acetate, and (6) 0.002 M DL-homocystine.

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<sup>1</sup> Holtzman Rat Company, Madison, Wisconsin.

<sup>2</sup> Serine-3 C<sup>14</sup> was purchased from the California Foundation for Biochemical Research on allocation by the United States Atomic Energy Commission.

The incubations were carried out for 2 hours in an oxygen atmosphere at 37° with constant shaking, the reaction being terminated by tipping in 0.5 ml of 20 per cent perchloric acid from the side arm. The carbon dioxide which was collected in the alkaline center well was precipitated and counted as BaCO<sub>3</sub>. The tissue was frozen in liquid air and pulverized. The extractions were carried out as previously described to yield acid soluble, lipid, nucleic acid, and protein fractions (1).

The nucleic acid fraction was hydrolyzed and the mixed purine bases were isolated according to the methods of LePage (4). Adenine and guanine were separated by paper chromatography by using the *tert*-butanol-HCl (5) and the *n*-butanol-H<sub>2</sub>O systems (6). The bases were eluted from the paper, counted, and the amounts of adenine and guanine determined spectrophotometrically by using the molar extinction coefficients of Markham and Smith (7).

The protein residue was washed, dried, and plated for counting. Samples of protein residue were hydrolyzed according to the procedure of Levy and Chung (8). Analysis of radioactivity in the individual amino acids was carried out, as described previously (1), after separation of the amino acids by paper chromatography, the *m*-cresol and phenol systems of McFarren (9) buffered at pH 8.4 and pH 12, respectively, being used.

All the determinations of radioactivity in these experiments were made on solid samples plated on tared planchets and counted in gas flow proportional counters for a sufficient period to obtain 5 per cent statistical accuracy. All the calculations are corrected for self-absorption and counting efficiency.

The results are expressed as counts per minute per mg of dry protein nucleic acid residue remaining after lipid extraction. In this study three separate experiments were performed in which the same results were obtained. A representative experiment is reported herein.

### Results

*Incorporation of Serine-3-C<sup>14</sup> into Protein*—The data in Table I demonstrate that the incorporation of labeled serine into the protein of the surviving uterus was stimulated by estrogen pretreatment to the same extent as had been found earlier for other amino acids (2, 10). Addition of no radioactive formate and glycine as cosubstrates reduced the total number of counts in the protein of both control and treated uterus almost proportionately. This can be explained by the endogenous synthesis of serine which diluted the serine-3-C<sup>14</sup>.

*Distribution of Radioactivity among Amino Acids of Hydrolyzed Protein*—Approximately 60 per cent of the incorporated radioactivity was accounted for as serine (Table II) in both types of tissue. Although the incorporation

of the radioactivity into methionine was also stimulated, it accounted for only a small portion of the total. This conversion was enhanced by the addition of homocystine (precursor for homocysteine) as acceptor for the methyl group in the formation of methionine. Unlabeled formate acted

TABLE I

*Effect of Estradiol on Incorporation of Serine-3-C<sup>14</sup> into Protein of Surviving Rat Uteri*

Data expressed as counts per minute per mg of isolated protein residue, system as under "Methods." The values represent duplicate flasks.

Non radioactive substrates	Control	Treated
None	7420 6050	11,150 10,360
Formate	4940 6050	9,180 8,820
Glycine	6620 6460	10,620 11,200
Formate + glycine	4860 4410	7,840 7,860
Acetate	7350 7490	11,400 10,560
Homocystine	6200 6120	12,080 11,460

TABLE II

*Distribution of Radioactivity Among Amino Acids of Hydrolyzed Protein*

System as under "Methods." The data are expressed as counts per minute per mg of protein residue. The values represent duplicate flasks.

Non radioactive substrates	Serine		Methionine	
	Control	Treated	Control	Treated
None	3860 3260	6300 4800	97 94	300 280
Formate	3020 3620	4950 5150	44 48	71 67
Glycine	3240 3420	6400 6000	86 78	310 260
Formate + glycine	1850 2120	3600 4620	12 20	38 34
Acetate	3820 3750	4200 5800	130 134	325 275
Homocystine	3820 3880	6050 6300	378 495	700 820

as a trap for the radioactive C<sub>1</sub> unit from the serine-3-C<sup>14</sup>, as indicated by a decreased number of counts in the methionine

*Production of CO<sub>2</sub> from Serine-3-C<sup>14</sup>*—In previous studies (1) the oxidation of the various radioactive substrates to CO<sub>2</sub> was not affected significantly by estrogen pretreatment. As shown in Table III, the production of carbon dioxide from serine-3-C<sup>14</sup>, however, was increased to a degree comparable to the effect of estrogen pretreatment on incorporation into protein. The addition of unlabeled formate as a cosubstrate acted as a pool to trap this C<sub>1</sub> unit, obscuring the action of the estrogen in the complete oxidation to carbon dioxide by this pathway. In support of the

TABLE III

*Effect of Estradiol on Production of CO<sub>2</sub> from Serine-3-C<sup>14</sup> by Surviving Uteri System as under "Methods"* The data are expressed as counts per minute per CO<sub>2</sub> per mg of protein. The values represent duplicate flasks

Non-radioactive substrates	Control	Treated
None	245	372
	257	391
Formate	204	174
	198	140
Glycine	262	304
	258	334
Formate + glycine	176	170
	167	170
Acetate	180	315
	194	352
Homocystine	203	365
	168	312

view, it was found that the specific activity of the formate pool in the flasks with control tissue was 1310 c p m per  $\mu$ mole, while that in the flask with estrogen-treated tissue was 2590 c p m per  $\mu$ mole. Thus, it appears that increased conversion of serine-3-C<sup>14</sup> to carbon dioxide is a reflection of the hormonal activation of serine metabolism, yielding formate which, as shown previously, is converted to CO<sub>2</sub> by an estrogen-insensitive reaction (1).

*Incorporation of Serine-3-C<sup>14</sup> into Lipide Fraction*—The incorporation of labeled serine into the lipide fraction (Table IV) was not stimulated by previous treatment with estrogen in contrast to earlier observations on the incorporation of radioactive formate and glycine (1). As indicated previously, the hormonal stimulation on the introduction of formate and glycine into lipides was mediated through the increased synthesis of serine which was then converted into the ethanolamine, choline, or glycerol

In the present experiments with labeled serine as substrate, the first reaction which is stimulated by estrogen has been bypassed and the subsequent reactions which incorporate serine into the lipid fraction are not affected by treatment with estrogen

*Incorporation of Serine-3-C<sup>14</sup> into Nucleic Acid Purines*—As is shown in Table V, treatment with estrogen caused a 5- to 6-fold stimulation in the incorporation of radioactivity from serine-3-C<sup>14</sup> into the adenine and guanine of the mixed nucleic acids. Similar results were obtained previously with formate-C<sup>14</sup> (1). The addition of non-radioactive formate as a trap

TABLE IV

*Effect of Estradiol on Incorporation of Serine-3-C<sup>14</sup> into Non-Volatile Lipide of Surviving Rat Uteri*

System as under "Methods." The data are expressed as counts per minute in lipid per mg of protein. The values represent duplicate flasks

Non radioactive substrates	Control	Treated
None	1305	1045
Formate	1200	900
	995	890
	920	825
Glycine	1125	1065
	1142	1020
Formate + glycine	978	955
	825	795
Acetate	1280	1018
	1280	1020
Homocystine	1070	1530
	1140	1520

for the labeled 1-carbon unit from serine caused a striking decrease in the amount of radioactivity incorporated into the purines. Thus the major effect of estrogen on this pathway must be upon the reactions that involve the formation of the 1-carbon unit from serine-3-C<sup>14</sup> and its subsequent metabolism. A similar result was found earlier with glycine-2-C<sup>14</sup> as the precursor (1). Apparently the formation of a 1-carbon unit from non-radioactive glycine is not sufficient to achieve a significant dilution of that derived from serine. Acetate had no effect on the metabolism of serine-3-C<sup>14</sup> in this experiment.

#### DISCUSSION

One of the early biochemical responses to estrogen stimulation of the rat uterus is an increased incorporation of amino acids into the protein (1, 10). As reported by Zamecnik and Keller (11) and Hoaglund (12),

who studied this incorporation process in liver preparations, a soluble enzyme system appears to be required for the activation of the carboxyl group of amino acids for the formation of hydroxamates or for the incorporation of amino acids into the protein of microsomes. The incorporation of pyruvate-2-C<sup>14</sup> into the protein and lipid and of acetate-1-C<sup>14</sup> into the lipid fraction of uterine segments is also accelerated by treatment with estrogen.<sup>3</sup> Berg (13), Popják and Tietz (14), and Langdon (15) have reported an acetate activation system similar to that for amino acid activation.

In the present experiments, treatment with estrogen accelerated to a

TABLE V  
*Effect of Estradiol on Incorporation of Serine-3-C<sup>14</sup> into Purines of Surviving Rat Uteri*

System as under "Methods" The values represent duplicate flasks

Non-radioactive substrates	C p m per $\mu$ mole guanine		C p m per $\mu$ mole adenine	
	Control	Treated	Control	Treated
None	390	2290	465	2058
	405	2770	320	2060
Formate	233	398	145	202
	250	328	126	176
Glycine	370	2050	440	1820
	470	2680	400	1930
Formate + glycine	236	350	60	175
	205	290	71	185
Acetate	474	3180	364	2060
	425	3080	435	2010
Homocystine	414	2340	378	1920
	510	2050	492	1725

comparable extent the incorporation of serine into protein and the cleavage of this amino acid to yield radioactive formate which readily equilibrated with an exogenous pool of non-radioactive formate. It seems likely that the accelerating effect of estrogen on both of these pathways may involve a common step in serine metabolism, namely, the activation of serine. Previous results which indicate a stimulating effect of estrogen on formate incorporation into nucleic acid purines, as well as on the synthesis of serine from this precursor, would suggest a role of the estrogen in the activation of this compound also.

It is of interest that the reactions discussed here, which involve amino acids (serine, glycine, alanine, lysine, and tryptophan), pyruvate, acetate, and formate, all concern carboxyl groups although the products and re-

<sup>3</sup> Unpublished data

actants are quite dissimilar. A common denominator by which estrogen might promote these processes in certain loci of the cell could therefore be the enhancement of those reactions which concern the activation of carboxyl groups. The manner in which estrogens play a part in such processes is under investigation.

The difference in magnitude of the estrogen effect on the incorporation of serine-3-C<sup>14</sup> into protein and into the purines of nucleic acids may reflect the action of estrogen at multiple sites along the pathway of purine biosynthesis in surviving uteri. This difference of response with a common precursor would seem to exclude an altered permeability as a major factor responsible for the estrogen effect on serine metabolism.

#### SUMMARY

1 The effect of 6 hours pretreatment with estradiol-17 $\beta$  on the incorporation of serine-3-C<sup>14</sup> into various metabolic components of the rat uterus was studied.

2 The incorporation into the protein and the production of carbon dioxide from the radioactive serine were stimulated to a comparable extent by treatment with estrogen.

3 Incorporation of radioactivity from the serine into the lipid fraction was not affected by the hormone.

4 Treatment with estrogen caused a 5- to 6-fold stimulation in incorporation of radioactivity into the purine bases.

5 The addition of non-radioactive formate as a trap for the labeled 1-carbon unit from the serine obscured the estrogen effect on the incorporation of radioactivity through this pathway.

6 The general pattern of estrogen influence on the incorporation of various precursors is discussed.

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# THE DETERMINATION OF INORGANIC PHOSPHATE AND CREATINE PHOSPHATE IN TISSUE EXTRACTS\*

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A number of procedures have been developed for the determination of inorganic orthophosphate (IP) and creatine phosphate (CP) in extracts of muscle or nervous tissue (1-6). Probably the simplest procedure in current use is that in which IP is determined in the presence of CP by the method of Lowry and Lopez (7) on one aliquot of extract, and IP plus acid-molybdate-labile organic phosphate is determined by the method of Fiske and Subbarow (8) on a second aliquot. The difference in phosphate concentrations given by the two methods is taken as a measure of the CP. Sometimes an accurate determination of IP by the Lowry-Lopez method is difficult because of the presence of substances in tissue extracts which retard the development of the reduced phosphomolybdate color<sup>1</sup>. Also, in extracts in which the concentration of IP is much greater than that of CP, a small error in the determination of IP or in the determination of IP plus CP can lead to a large error in the estimation of CP. In the procedure to be reported here the last mentioned disadvantage of the Lowry and Lopez procedure is overcome, since IP and CP are determined successively on the same aliquot of extract during a continuous run in a spectrophotometer. The procedure is essentially a modification of that of Fiske and Subbarow. The IP of an aliquot is first determined in weak acid (pH 2.3) with the aid of added  $\text{Cu}^{++}$  to increase the rate of development of the reduced phosphomolybdate color<sup>2</sup>. Strong acid is then added to the aliquot and the extra IP formed during the subsequent rapid hydrolysis of CP is determined by the further increase in color.

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<sup>1</sup> This difficulty may be overcome by adding to the extracts a small amount of  $\text{Cu}^{++}$ , which greatly accelerates the color development (9, 10).

<sup>2</sup> The use of  $\text{CuSO}_4$  was first suggested by Dr. C. R. Hanes in 1952 (personal communication). Prior to the development of the present method, Dr. O. H. Lowry and his associates had already found that  $\text{CuSO}_4$  greatly increases the rate of color development in the Lowry and Lopez method and also in a modified Fiske and Subbarow method at pH 2.0. More recently other workers have used  $\text{CuSO}_4$  in the Lowry and Lopez method (9, 10).

### *Materials and Methods*

*Reagents and Standards*—All inorganic reagents and standards were of analytical grade. The 1-amino-2-naphthol-4-sulfonic acid was a Fisher "certified reagent." The creatine phosphate used in most cases was the sodium salt, obtained from the Sigma Chemical Company, containing less than 2 per cent of its phosphate as IP. In some of the early experiments a solution of the potassium salt of creatine phosphate was used. This was prepared by mixing the calcium salt (purified from rabbit muscle by Dr. Helen Graham) with an equivalent amount of potassium oxalate in solution and centrifuging the calcium oxalate formed. The solutions of CP ranged from 0.004 to 0.009 M. Aliquots were kept frozen until the day of use. 0.3 M solutions of perchloric acid (PCA) and 0.004 M solutions (1 gm of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  per liter) of copper sulfate were used. The Fiske and Subbarow reducing reagent was as follows: a solution containing 0.2 per cent 1-amino-2-naphthol-4-sulfonic acid, 1.2 per cent  $\text{Na}_2\text{SO}_3$ , and 12.0 per cent  $\text{NaHSO}_3$ . This was stored in a brown glass bottle in a refrigerator. A 2.5 per cent solution of  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$  was employed. The Fiske and Subbarow mixture (FSM) was a mixture of 1 volume of reducing reagent and 2 volumes of ammonium molybdate solution. This mixture was made fresh on the day of use and kept in a test tube in an ice bath. The sulfuric acid was a 5.0 N solution. The inorganic phosphate standards were 0.003 and 0.007 M solutions of  $\text{KH}_2\text{PO}_4$ .

*Preparation of Tissue Extracts*—The tissue sample to be analyzed was frozen in liquid nitrogen and taken to a cold room at  $-20^\circ$ , where it was weighed in the frozen state on a torsion balance. In the case of muscle preparations which had been suspended in Krebs-bicarbonate medium prior to freezing, any adhering frozen droplets of medium were scraped off before the weighing. Occasionally a tissue sample was extracted immediately after being weighed, but usually it was stored for 20 to 30 hours at  $-20^\circ$  before being extracted.

The extraction procedure was carried out in a cold room at  $2^\circ$ . The frozen tissue sample, usually weighing from 50 to 100 mg, was placed in a stainless steel, 50 ml centrifuge cup (obtained from Ivan Sorvall, Inc.), which was encased in aluminum foil and held upright in a container filled with fragmented dry ice.<sup>3</sup> A few ml of liquid nitrogen were poured onto the tissue in the cup and allowed to boil off, and the solidly frozen tissue was then pulverized with the aid of a special plunger. The plunger consisted of a stainless steel head, machined to fit snugly into the lower half of the cup, a stainless steel shaft, and a cylindrical brass handle with a

<sup>3</sup> If the cup is not encased in aluminum foil, pieces of dry ice may adhere to the outside of the cup when it is removed from the dry ice container following pulverization of the tissue. This may cause the PCA added to the cup to freeze.

flat top The head of the plunger, previously chilled to dry ice temperature, was inserted into the cup, so that it rested on the frozen tissue, and the flat top of the handle was struck with a hammer Several sharp blows were usually sufficient to pulverize the tissue into a fine powder The plunger was then twisted and removed from the cup Any powdered tissue adhering to the head of the plunger was scraped back into the cup with a small stainless steel spatula which also had been previously chilled to dry ice temperature

After pulverization of the tissue, the stainless steel cup was removed from the casing of aluminum foil in the dry ice container and placed in an ice bath 1 ml of ice-cold 0.3 M PCA was immediately added<sup>4</sup> and the powdered tissue and PCA were stirred intermittently with a glass rod for 20 minutes, a period found to be adequate for complete extraction of acid-soluble phosphates 5 ml of H<sub>2</sub>O were then added, giving a final PCA concentration of about 0.05 M and, after a brief mixing, the contents of the cup were poured into a chilled 12 ml thick walled conical centrifuge tube This was centrifuged in the cold room at about 2500 r p m for 10 minutes and the supernatant extract was decanted into a test tube in an ice bath

*Analytical Procedure*—Extracts were analyzed for IP and CP within 1 hour after the end of the extraction procedure Hydrolysis of CP was insignificant over this period as long as the extracts were kept at 0° in an ice bath To a 2 ml portion of the extract were added 80 c mm of CuSO<sub>4</sub> solution<sup>5</sup> CuSO<sub>4</sub> solution was also added in the same proportion to a blank solution (0.05 M PCA) and a standard solution (phosphate standard diluted 60-fold with 0.05 M PCA) contained in tubes in the same ice bath with the extract

40 c mm of chilled FSM were added to each of four microcells of approximately 500 c mm capacity set in a microcell carrier of a Beckman DU spectrophotometer fitted with a pinhole diaphragm<sup>6</sup> Then in succession blank solution was added to the first cell, standard solution to the second,

<sup>4</sup> 0.3 M trichloroacetic acid may also be used In the present work PCA was preferred because analyses for adenine nucleotides by the method of Kalckar (11) were often carried out on aliquots of the extracts, and this method cannot be used on trichloroacetic acid extracts

<sup>5</sup> In the present experiments CuSO<sub>4</sub> was not added to that portion of the extract to be used for the determination of adenine nucleotides by the method of Kalckar (11), since cupric ions interfere with this enzymatic method

<sup>6</sup> All additions of solutions were made with constriction pipettes of the Lang-Levy type (12) Microcells of fused silica were 10 mm deep, 2.5 mm wide, and 25 mm high Microcells, the microcell carrier, and the pinhole diaphragm for the spectrophotometer were obtained from Pyrocell, Inc., New York The microprocedure used here can be easily modified into a macroprocedure by proportionally increasing the volumes of all solutions used In the macroprocedure a photoelectric colorimeter may be used in place of the Beckman spectrophotometer

and extract to the third and fourth. All these additions were 200 c mm in volume and were made with the same constriction pipette, which was rinsed once with each solution before being used for delivery of that solution. After each addition to a microcell, its contents were quickly mixed with a small flat Lucite rod with a foot. The intervals between additions were 15 to 20 seconds. The cell carrier was placed in the spectrophotometer and optical density readings (with the blank set at zero) were made at a wave length of 660  $\mu$  on the standard and extracts at 2, 3, 4, and a minute intervals from the time of addition of the FSM. During this first phase of the analysis, which is for the determination of IP, the pH of the solutions in the microcells is approximately 2.3.

Immediately following the readings at the 5 minute interval, 25 c mm of 5 N  $\text{H}_2\text{SO}_4$  were added to each microcell, and the contents of each were mixed with a Lucite rod after each addition. Optical density readings were then made at 7, 8, 9, and 10 minute intervals from the time of addition of the FSM and then at less frequent intervals to 30 minutes, by which time essentially all of the CP was hydrolyzed.

For analysis of test solutions containing mixtures of CP and IP, the same procedure was used as was employed with tissue extracts. Such solutions were freshly made up in 0.05 M ice-cold PCA and kept at 0° until analyzed. In some experiments with test solutions, water was used in the reference cell of the spectrophotometer and the optical density of the blank, as well as that of the standard and test solutions, was followed.

The temperature of the room in which the spectrophotometric analyses were made ranged from 24–27°. The temperature of the solutions in the microcells rose several degrees above room temperature during the course of an analysis.

#### RESULTS AND DISCUSSION

*Test Solutions of CP and IP*—Fig. 1 is a plot of the changes in optical density of a blank solution, two IP standards, and two test solutions containing CP during the course of analysis. Due to the presence of the added  $\text{Cu}^{++}$ , development of the reduced phosphomolybdate color in the IP standards is complete within 2 minutes. The ratio of the optical densities of the two IP standards is the same as the ratio of their concentrations from the 2 to 30 minute reading, that is, Beer's law is satisfied at any time during the course of the analysis, despite the changes in optical density which occur. In the case of the IP standards there is essentially no change in optical density from the 2 to 5 minute reading. The addition of  $\text{H}_2\text{SO}_4$  (to give approximately 0.5 N) produces an immediate fall due to dilution. The optical density then rises somewhat during the next few minutes and then continues to rise much more slowly and linearly with time until the end of the experiment.

In the case of CP and CP + IP solutions, the optical density increases almost linearly with time from 2 to 5 minutes as a result of the hydrolysis of a small fraction (about 10 per cent) of the CP to IP and creatine during this period. An accurate measure of the optical density due to the IP initially present in these solutions may be obtained by extrapolating the 2 to 5 minute curve to zero time (dash lines in Fig 1). The extrapolated value for the optical density of the CP + 0.003 M IP solution (0.168) is equal to the sum of that for the CP solution (0.016) plus the optical density

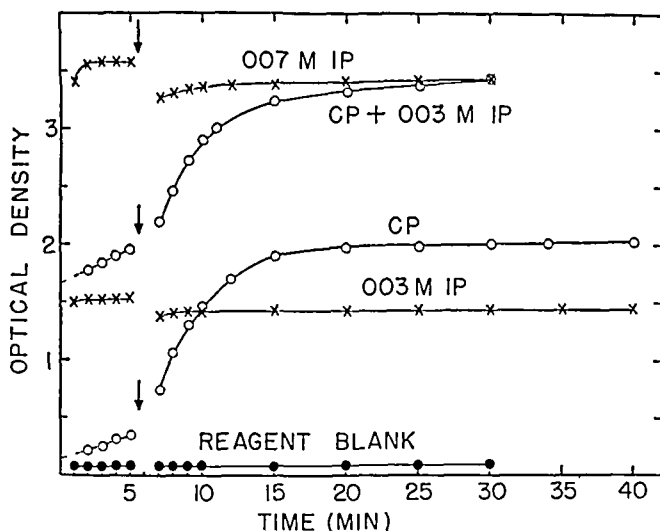


Fig 1 Changes in optical density during course of analysis. The four upper curves are already corrected for optical density of the reagent blank. The solutions were mixed with FSM at zero time and 5 N  $\text{H}_2\text{SO}_4$  was added at the arrows. The IP standards at the concentrations shown were diluted 60-fold with 0.05 M PCA before use. Solutions of CP (approximately 0.004 M) and CP + IP were similarly diluted. About 8 per cent of the phosphate in the CP used was IP.  $\text{CuSO}_4$  was added to all solutions as described in the text.

(at 5 minute reading) for the 0.003 M IP standard (0.152). This indicates that the presence of CP does not interfere with the determination of IP. It may also be noted that the optical density of the CP + IP solution at the 30 minute reading is essentially equal to that of the CP solution plus that of the 0.003 M IP standard at that time.

The addition of  $\text{H}_2\text{SO}_4$  to the solutions containing CP greatly accelerates the hydrolysis of CP, thus a rapid rise in optical density ensues as IP is liberated and reacts to form reduced phosphomolybdate (Fig 1). Although the optical densities of the CP and CP + IP solutions are still increasing slowly at 30 minutes, the CP is essentially all split by this time. The continued slow increase after 25 to 30 minutes is not due to further liberation of IP, but corresponds to the similar slow increase in optical

density of the standards. This is clearly shown if a plot is made of the "apparent IP" present during the course of an analysis, where "apparent IP" at each time interval is obtained by dividing the optical density of the standard into that of the solution containing CP and multiplying by the proper factor to convert to micromoles per ml of original solution. Such a plot, made from the data in Fig 1 and shown in Fig 2, clearly indicates that the hydrolysis of CP is essentially complete at 30 minutes (25 minutes after the addition of  $\text{H}_2\text{SO}_4$ ), for by this time the curves, which rise in exponential fashion after the addition of  $\text{H}_2\text{SO}_4$ , have reached maxima. If it is assumed that the reaction which limits the rate of rise of these

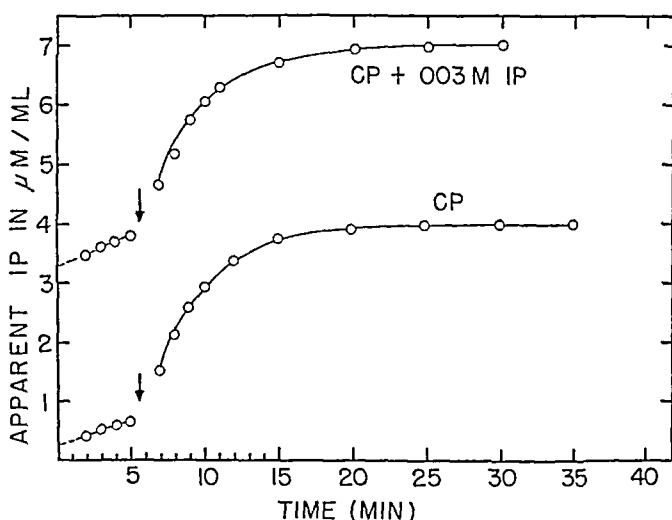


FIG 2 Data from Fig 1 plotted to give "apparent IP" in CP and CP + IP solutions at intervals during course of analysis. "Apparent IP," determined as indicated in the text, is in micromoles per ml of original solution before dilution with PCA prior to analysis. The arrows indicate the time of addition of  $\text{H}_2\text{SO}_4$ .

curves is the hydrolysis of CP, then the half time for this hydrolysis is from 2.5 to 3 minutes (Fig 2 and Table II).

**Correction Factor for CP Concentration**—The extrapolated zero time value in Fig 2 shows that the solution of CP tested (potassium salt preparation) contained 0.3  $\mu\text{moles}$  of IP per ml. The "apparent IP" at the end of the determination was 4.0  $\mu\text{moles}$  per ml, giving an "apparent CP" concentration by difference of 3.7  $\mu\text{moles}$  per ml. However, when this CP solution was subjected to mild acid hydrolysis sufficient to convert all of the labile organic phosphate to IP (2 hours at  $40^\circ$  or 5 minutes at  $100^\circ$  in 0.05 M PCA) the total IP was found to be 4.24  $\mu\text{moles}$  per ml, indicating that the actual CP concentration was 3.94  $\mu\text{moles}$  per ml, a value about 6.5 per cent above the "apparent CP" concentration.

In a series of about twenty experiments with solutions of CP (both pota-

sum and sodium salts) in which the actual CP concentration and the "apparent CP" concentration were determined as described above, the value of the former ranged from 5.5 to 6.8 per cent above that of the latter and averaged close to 6 per cent above it.

The reason why the present analytical procedure gives a value for CP which is somewhat low is that the extinction coefficient on the basis of phosphate concentration for the reduced phosphomolybdate complex developed in the first step of the procedure at pH 2.3 and then further acidified with  $\text{H}_2\text{SO}_4$  is greater than that of the complex developed from the begin-

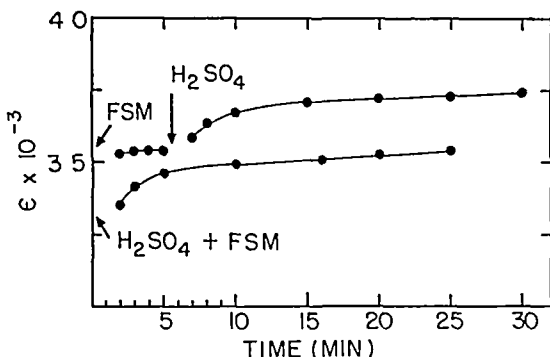


FIG 3 Molar extinction coefficient ( $\epsilon$ ), on basis of IP, in procedure in which  $\text{H}_2\text{SO}_4$  is added about 5½ minutes after FSM (upper curve) and in Fiske and Subbarow method in which  $\text{H}_2\text{SO}_4$  is added before FSM (lower curve). In each procedure 200 c mm of the same standard solution (0.00011 M IP in 0.05 M PCA +  $\text{CuSO}_4$ ) were first added to microcells. Volumes of FSM and 5 N  $\text{H}_2\text{SO}_4$  were, respectively, 40 and 25 c mm. The arrows indicate the additions. In the experiment for the lower curve  $\text{H}_2\text{SO}_4$  was added and mixed just before FSM was added at zero time.

ning in  $\text{H}_2\text{SO}_4$ . This difference in extinction coefficient is shown in Fig. 3. The upper curve shows the molar extinction coefficient of a phosphate standard during the course of analysis in which  $\text{H}_2\text{SO}_4$  was added 5½ minutes after FSM. The lower curve shows the extinction coefficient of the same standard when  $\text{H}_2\text{SO}_4$  was added just prior to FSM and would therefore be very similar to that given by IP formed by the rapid hydrolysis of CP following the addition of  $\text{H}_2\text{SO}_4$  in the present procedure. The molar extinction coefficient 25 minutes after the addition of  $\text{H}_2\text{SO}_4$  in the upper curve is 3750, whereas it is 3540 at the 25 minute reading in the lower curve. This 6 per cent difference accounts for the difference found between "apparent CP" by the present procedure and actual CP in test solutions.

Because of this difference in "apparent CP" and actual CP, all "apparent CP" values obtained with tissue extracts should be multiplied by 1.06 to obtain a "corrected CP" value.<sup>7</sup>

<sup>7</sup> If a smaller correction factor is desirable, the analysis may be carried out at a wave length of 1000 m $\mu$  rather than 660 m $\mu$ . At this higher wave length there is less



*Tissue Extracts*—Fig 4 shows typical curves for optical density again time during the course of analysis of extracts of muscle and nerve. The "normal auricle" was an isolated left auricle of a guinea pig which had been driven electrically at 1 beat per second for about 30 minutes in oxygenated Krebs-bicarbonate solution at 37°. The "poisoned auricle" was similar to the "normal" except that it had been exposed to  $5 \times 10^{-6}$  *l*-strophanthin for about 20 minutes. This cardiac glycoside had first produced an increase in force of contraction, but by the time the auricle was frozen for analysis, the force had decreased about 80 per cent as a result of poisoning by an excess of the drug. Fig 4 indicates that this decrease in force

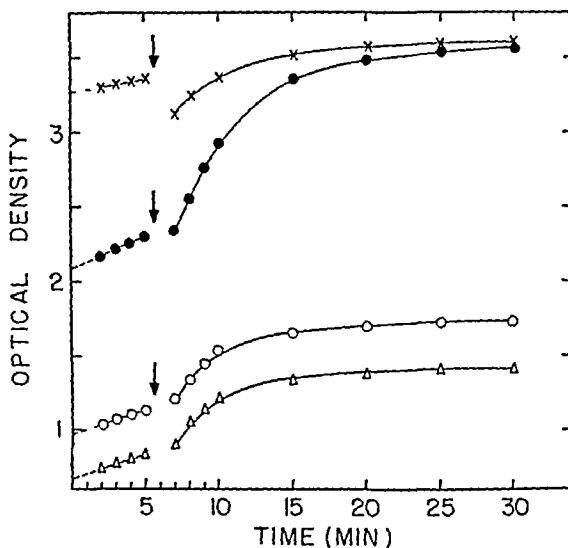


FIG 4 Optical density changes during course of analysis for IP and CP in PCA extracts of different tissues ●, guinea pig auricle, ×, guinea pig auricle after poisoning with excess *l*-strophanthin, ○, smooth muscle of rabbit stomach, Δ, frog sciatic nerve. The arrows indicate the additions of 5 N  $H_2SO_4$ .

associated with a marked decrease in CP concentration and increase in IP concentration.

The applicability of this analytical procedure to smooth muscle and nerve is also shown by the lower curves in Fig 4. The smooth muscle analyzed was a strip of muscle separated from the mucosa of rabbit stomach and incubated for 30 minutes in oxygenated Krebs-bicarbonate solution at 37°. The nerve analyzed was frog sciatic nerve. Two nerves were

discrepancy between the extinction coefficient of the reduced phosphomolybdate complex developed in the first step of the procedure and that developed after addition of  $H_2SO_4$ , and as a result of this the correction factor is about 1.02 rather than 1.06. The disadvantage of using  $\lambda$  1000  $m\mu$  is that the extinction coefficient for the reduced phosphomolybdate complex at any time during the course of analysis is only about 80 per cent as great as it is at  $\lambda$  660  $m\mu$ .

pooled and kept in oxygenated frog Ringer solution for about 10 minutes prior to freezing

In the analysis of extracts of muscle and nervous tissue the IP concentration and the "apparent CP" concentration are obtained by the technique described for test mixtures of IP and CP, with the optical density (extrapolated) at zero time and the optical density at the 30 minute interval. The optical density readings at intervals between the time of addition of the  $\text{H}_2\text{SO}_4$  and the 30 minute interval are not required for the determination of the concentration of "apparent CP," but these intermediate readings should be made in preliminary experiments on each type of muscle or nervous tissue studied to ascertain whether the organic phosphate hydrolyzed during the second phase of the analysis behaves like creatine phosphate. If it does, a plot of "apparent IP" against time following the addition of  $\text{H}_2\text{SO}_4$  (as in Fig. 2) should give an exponential curve with a half time similar to that of CP (2.5 to 3 minutes). This requirement was found to be satisfied in the case of all tissue extracts reported on in the present work.

The results of analyses on the extracts of three different types of mammalian muscle and of frog sciatic nerve are shown in Table I. In all experiments CP concentrations were obtained by multiplying the "apparent CP" concentrations by the correction factor, 1.06, previously discussed.

With three extracts of guinea pig auricles recovery experiments were carried out on CP. A known concentration of this substance was added to a portion of each extract, and the concentrations of CP in the original extract and in the portion with added CP were determined. The extra CP shown by analysis amounted to 99, 100, and 101 per cent of the added CP in the three experiments.

*Variations in  $\text{CuSO}_4$  Concentration*—In the absence of  $\text{CuSO}_4$  the development of the reduced phosphomolybdate color after addition of FSM to standard IP solutions in 0.05 N PCA is only about 60 per cent complete in 2 minutes and is still several per cent short of completion in 5 minutes. In the presence of the usual concentration of  $\text{CuSO}_4$  (final concentration of 0.15 mM) color development due to IP initially present in standard solutions or extracts is over 90 per cent complete in 1 minute and essentially complete in 2 minutes (Figs. 1 and 4). Increasing the concentration of  $\text{CuSO}_4$  2- to 5-fold does not give any appreciable further increase in the rate of development. The effect of  $\text{CuSO}_4$  in the present procedure differs from that observed in the Lowry and Lopez procedure (9) in that the presence of  $\text{Cu}^{++}$  in the present method does not result in an enhancement of the extinction coefficient of the reduced phosphomolybdate complex. In the Fiske and Subbarow procedure  $\text{CuSO}_4$  neither increases the rate of color development nor increases the extinction coefficient.

*Variations in FSM Concentration*—In the standard procedure developed

here 40 c mm of FSM are added to 200 c mm of solution, approximately 0.05 N with respect to PCA. Due to the  $\text{Na}_2\text{SO}_3$  and  $\text{NaHSO}_3$  of the FSM the addition immediately increases the pH of the solution from about 1.4 to about 2.3. This latter pH level is close to the pK of  $\text{H}_2\text{SO}_3$  (about 1.8), thus the solution is well buffered during the first part of the procedure during which time IP is determined. The effects of variations in the amount of FSM added have not been extensively tested. However, it should be noted that the addition of 50 c mm rather than 40 c mm of FSM de-

TABLE I

*Inorganic Phosphate, Creatine Phosphate, and Adenyl Pyrophosphate (APP) in Different Types of Mammalian Muscle and in Frog Nerve*

Auricles, stomach muscle, and frog nerve were frozen for analysis after incubations *in vitro* as described in the text. Skeletal muscle (rat gastrocnemius) was frozen immediately after excision.

Tissue*	$\mu\text{moles per gm tissue (wet weight)}$		
	IP	CP	APP†
Guinea pig left auricle	5.91 $\pm$ 0.31	4.35 $\pm$ 0.17	5.44 $\pm$ 0.14
Rabbit stomach muscle	2.6	1.5	2.8
Rat skeletal muscle	5.1	18.4	6.7
Frog sciatic nerve	2.0	1.6	1.9

\* The values for guinea pig auricles are mean values ( $\pm$  standard error of the means) obtained with extracts from eleven auricles. The values for other tissue are from representative experiments on single extracts. The values for skeletal muscle were kindly supplied by Dr. Ethel Ronzoni.

† APP represents labile phosphate of ATP and adenosine diphosphate. This was determined enzymatically by the method of Kalekar (11) in the case of auricle and stomach muscle, by IP liberation during a 15 minute hydrolysis at 100° in 1 N  $\text{H}_2\text{SO}_4$  in the case of nerve, and by chromatographic separation of ATP and adenosine diphosphate followed by spectrophotometric analysis in the case of skeletal muscle (13).

creases the rate of hydrolysis of CP in the first part of the procedure about 50 per cent. This slower splitting of CP appears to be a result of the higher pH (about 2.5) obtained with the 50 c mm addition. Since too rapid splitting of CP may lead to less accurate determinations of IP, it is recommended that 50 c mm of FSM be used in place of 40 c mm when the procedure is carried out at room temperatures above 30°. While the larger amount of FSM decreases the rate of CP hydrolysis in the first part of the procedure, it increases the rate of CP hydrolysis in the second part of the method (after addition of  $\text{H}_2\text{SO}_4$ ) more than 50 per cent, probably because of the higher molybdate concentration present.

In connection with the catalytic effect of molybdate on CP hydroly-

it was found that an approximately 5-fold increase in the ammonium molybdate concentration (to a final concentration of 1.25 per cent) did not appreciably increase this rate during the first part of the present procedure. This is somewhat surprising in view of the finding of Weil-Malherbe and Green (14) that 1 per cent ammonium molybdate greatly increases the rate of hydrolysis of CP at 0° at pH levels similar to those in our experiments.

TABLE II

*Hydrolysis of Different Phosphate Esters and Anhydrides in Standard Procedure*

All compounds were tested at a final concentration ranging from 0.05 to 0.15 mM. The room temperature at time of testing was from 24–27°. The change in optical density due to IP liberation was used to determine extent of hydrolysis.

Compound*	Hydrolysis during 1st part of procedure (5 min at pH 2.3)	Hydrolysis during 2nd part of procedure (25 min in 0.5 N H <sub>2</sub> SO <sub>4</sub> )	Half time for hydrolysis in 2nd part of procedure
	<i>per cent</i>	<i>per cent</i>	<i>min</i>
Creatine phosphate†	8–12	100	2.5–3.0
Deoxyribose-1-phosphate	100		
Acetyl phosphate	58	100	<1
Ribose-1-phosphate	8	100	1
Inorganic pyrophosphate	0	7	
Adenosine triphosphate	0	1	
Glucose-1-phosphate	0	2	

\* The following compounds were not detectably hydrolyzed in either part of the procedure: adenosine diphosphate, adenylic acid, glucose 6-phosphate, fructose-1-phosphate, fructose-6-phosphate,  $\alpha$ -glycerol phosphate, phosphopyruvate, diphosphopyridine nucleotide, triphosphopyridine nucleotide. Deoxyribose-1-phosphate and ribose-1-phosphate were kindly supplied by Dr Morris Friedkin, and acetyl phosphate and phosphopyruvate by Dr Arthur Kornberg. All other organic phosphates were obtained from the Sigma Chemical Company or the Pabst Laboratories.

† The differences in the rate of hydrolysis of CP in different experiments were probably due to variations in room temperature.

*Behavior of Various Phosphate Compounds*—A number of phosphate esters and anhydrides besides CP were tested in the present procedure (Table II). Deoxyribose-1-phosphate is hydrolyzed completely within the first 2 or 3 minutes, and therefore its presence would interfere with the analysis of IP. The rate of hydrolysis of acetyl phosphate in the first part of the procedure is also somewhat too rapid to permit an accurate analysis of IP in its presence. However, by reducing the molybdate concentration of the FSM to one-half, the rate of hydrolysis of acetyl phosphate may be reduced approximately one-half, thus permitting a fairly accurate IP analysis. Ribose-1-phosphate is hydrolyzed in the first part of the procedure at about the same rate as CP. Its presence in significant concentra-

tions would not interfere with the determination of IP, but would certainly lead to inaccuracies in the determination of CP. Inorganic pyrophosphate is hydrolyzed 7 per cent during the second part of the procedure, and at high concentration would interfere with the determination of CP. On the other hand, only about 1 per cent of the pyrophosphate of adenosine triphosphate (ATP) is liberated during this part of the method. Because the degree of hydrolysis of ATP is so small, no corrections have been made for it in the determinations of CP of tissue extracts reported on in this paper. Of the other compounds tested only glucose-1-phosphate undergoes a detectable degree of hydrolysis (about 2 per cent) during the second part of the procedure.

#### SUMMARY

1 The method of Fiske and Subbarow has been modified so that inorganic phosphate and creatine phosphate can be determined successively on the same aliquot of extract during a continuous run in a spectrophotometer. Inorganic phosphate is first determined at pH 2.3 with the aid of added  $\text{CuSO}_4$  to increase greatly the rate of development of the reduced phosphomolybdate color. Strong acid is then added to hydrolyze creatine phosphate, and the extra color developed gives a measure of this compound.

2 Applications of this procedure to the determination of inorganic phosphate and creatine phosphate in extracts of muscle and nerve are described.

3 A simple and convenient apparatus for pulverizing small samples of frozen tissue prior to extraction is described.

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# EFFECTS OF SOME DIETARY FACTORS ON THE METABOLISM OF FATTY ACIDS IN LIVER PREPARATIONS\*

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Previous results indicated that the oxidation of isotopic long chain fatty acids in isolated preparations of liver (1) and other tissues (2) is enhanced by choline, administered *in vivo* to rats on low casein diets. The diets used in these experiments contained little methionine (a major biological precursor of both choline and cystine) and were practically free of cystine. A study of the effects of supplementing such, or similar, diets with cystine seemed of interest, in view also of other findings in the literature. Indeed, fatty infiltration of the liver is more severe when cystine is added to high fat, low protein diets (3). On the other hand, a massive liver necrosis occurs frequently with certain diets which are deficient in S-containing amino acids (4). Since the latter condition is prevented by administration of cystine, as well as of tocopherol (5), the effects of supplementing the deficient diets with this vitamin were also studied. Both the  $\text{CO}_2$  production from isotopic palmitate or stearate, added to liver homogenates, and the incorporation of  $\text{C}^{14}$ -acetate into long chain fatty acids of liver slices were investigated.

In spite of considerable variations and some discrepancies, preliminary results (6) strongly suggested that cystine and tocopherol enhance the oxidation as well as the synthesis of fatty acids, whereas only fatty acid oxidation was stimulated by choline, administered terminally to the animals. These tentative conclusions are corroborated by the results of additional experiments and by a statistical analysis of the previous and more recent data. A comprehensive report of our study is presented here.

## EXPERIMENTAL

Male albino rats (Rockland Farms, initial weight, 50 to 70 gm) were maintained for various lengths of time (mostly 2 to 4 weeks) on one of the experimental diets (Table I) without or with added supplements. These included L-cystine, 1.0 gm,  $\alpha$ -tocopherol, 0.025 gm, DL-methionine, 1.0 gm, choline chloride, 0.5 gm per 100 gm of diet. To enhance the severity

\* Aided by the United States Atomic Energy Commission under a contract (No. AT(40-1)-1638). Isotopic compounds were obtained from commercial sources under allocation from the United States Atomic Energy Commission.

of choline deficiency in the experiments in which the effects of terminal injections of choline were studied, 1.5 gm of guanidoacetic acid (GAA) per 100 gm of diet were added to the choline- and cystine-deficient diet. In these experiments, choline chloride (0.25 mmole per 100 gm of rat) was administered in two intramuscular injections, 2 hours and 1 hour before death. The rats were killed by decapitation, and the livers were removed, chilled, and either homogenized (Potter-Elvehjem homogenizer) or sliced free hand.

In each experiment, liver preparations from one or more control rats,

TABLE I  
*Composition of Experimental Diets\**

Diet No	$\alpha$ Protein†	Casein	Crisco	Cod liver oil	Estimated content in S-containing amino acids‡
	per cent	per cent	per cent	per cent	per cent
53	8		4	1	0.11
55	15		4	1	0.20
26		5	4	1	0.19
47		8	4	1	0.31
52	8		30	2	0.11
54	15		30	2	0.20
28		5	30	2	0.19
46		8	30	2	0.31

\* In addition, all diets contained salt mixture (U S P XII) 4 per cent, Ruffer 2 per cent, dextrin and sucrose in equal amounts to make 100 parts. 1 ml of a solution of B vitamins (1) was incorporated in 100 gm of diet.

† Obtained from soy beans.

‡ Computed as methionine.

maintained on a deficient diet (unsupplemented, or with added GAA), were incubated simultaneously with similar preparations from rats which had been maintained, generally for the same length of time, on the same diet with added supplements. In addition to the experimental flasks (two to four flasks for each liver preparation), one or two "blank flasks" were prepared exactly as the experimental flasks, except that the tissue was placed in a boiling water bath for 10 minutes before the isotopic substrate was added. The flasks were shaken for 3 hours in air in a Warburg bath at 37°.

In the experiments on fatty acid oxidation, the incubation medium contained, in a total volume of 6 ml of Ca-free Ringer-phosphate (pH 7.4),<sup>3</sup> 3 mg each of penicillin and dihydrostreptomycin, 12  $\mu$ moles of adenosine

<sup>1</sup> The following abbreviations are used: GAA, guanidoacetic acid, ATP, adenosine triphosphate, CoA, coenzyme A, DPN, diphosphopyridine nucleotide.

triphosphate (ATP), 300 to 600 mg of homogenized tissue, and 1 to 2  $\mu$ moles (1 to 2  $\mu$ c) of palmitate-1- $C^{14}$ , or, less frequently, stearate-1- $C^{14}$ . The  $C^{14}O_2$  produced during the incubation was collected and measured as described (1).

In the experiments on fatty acid synthesis, 500 to 700 mg of slices, 3 mg each of penicillin and dihydrostreptomycin, and acetate-1- $C^{14}$  (2  $\mu$ moles, 1  $\mu$ c) were incubated in 5 ml of Ringer-phosphate, containing Ca and Mg (pH 7.4). At the end of the incubation period,  $C^{14}$  was determined on the respiratory  $CO_2$  and on the fatty acids and cholesterol extracted from the tissue. The contents of the main compartments of two flasks were pooled and saponified with NaOH in water-ethanol. The unsaponifiable matter was extracted with petroleum ether from a medium containing approximately 0.1 N NaOH in 50 per cent ethanol. The combined petroleum ether extracts were washed with an aqueous solution of non-isotopic acetate, and, after evaporation of the solvent, cholesterol was precipitated with digitonin from an acetone-ethanol-water solution (7). After extraction of the unsaponifiable matter, the soap solution was freed of ethanol by evaporation, acidified, and extracted with ethyl ether. The ether was evaporated, and the residue, dried *in vacuo* at 55°, was taken up in petroleum ether. The solution was filtered through asbestos, the solvent was removed, and the total fatty acids were weighed and redissolved in chloroform. Aliquots of this solution were brought to a standard weight by addition of egg lipides, plated, and counted. In a few experiments, the long chain fatty acids, after saponification and extraction of the unsaponifiable matter, were precipitated with  $CuSO_4$  and  $Ca(OH)_2$ , according to Lehninger and Smith (8), and the precipitate was washed repeatedly by centrifugation with a solution of non-isotopic Na acetate. After acidification, the fatty acids were extracted with ethyl ether, plated, and counted. When either procedure was applied to the "blank flasks," only negligible amounts of radioactivity were found in the recovered fatty acids and cholesterol.

On aliquots of the liver homogenates, total N (9), total lipides (weight of the chloroform extract (10)), and lipide P (11) were determined. The differences between total lipides and total phospholipides (lipide P  $\times$  25) were designated as liver "fat," these values include cholesterol and other unsaponifiable substances in addition to triglycerides. In the experiments on fatty acid synthesis, total N was determined on separate samples of liver slices.

### Results

In each experiment, the " $C^{14}O_2$  ratios" (or the " $C^{14}$ -fatty acid ratios") were calculated, *i.e.*, the ratios of the values obtained on the livers of the



rats receiving a given supplement to the corresponding value obtained, in the same experiment, on the livers of the control animals, fed the deficient diet. For the sake of brevity, only the medians and the means of the

TABLE II  
*Effects of Supplementing Various Diets with Cystine on Fatty Acid Oxidation in Liver Homogenates*

The substrate was stearate-1- $C^{14}$  or palmitate-1- $C^{14}$

Diet No *	Dietary protein	Average change in liver "fat"†	C <sup>14</sup> O <sub>2</sub> ratios‡			
			Per mg liver N		Whole liver§	
			Medians	Means	Medians	Means§
Low fat diets						
53 (5)	α-Protein	+566	2 05	} 2 53** ± 0 51	2 60	} 2 72** ± 0 41
55 (9)	"	+1034¶	2 22		2 57	
26 (12)	Casein	+230¶	1 36	} 1 55** ± 0 20	1 51	} 1 77** ± 0 31
47 (7)	"	+582¶	1 42		1 59	
High fat diets						
52 (3)	α-Protein	+93	1 79	} 2 25** ± 0 29	1 84	} 2 45** ± 0 37
54 (6)	"	+117	2 75		2 69	
28 (6)	Casein	-227	1 61	} 3 23** ± 0 97	1 47	} 3 31** ± 1 07
46 (6)	"	-387	1 29		1 22	

\* No of experiments in parentheses

† The "fat" content of the livers of rats on the cystine-supplemented diet, less that of the livers of rats on the unsupplemented diet. "Fat" = total lipides (by weight), less total phospholipides (lipide P  $\times$  25)

‡ Ratios of  $C^{14}O_2$  produced by the livers of rats on the cystine-supplemented diet to the  $C^{14}O_2$  produced, in the same experiment, by the livers of the control rat on the unsupplemented diet

§ Values adjusted to 100 gm of body weight

|| The figures preceded by  $\pm$  are the standard errors of the means

¶ Significantly different from 0 ( $P < 0.05$ ). The unmarked values are not significantly different from 0 ( $P > 0.05$ )

\*\* Significantly different from 1 ( $P < 0.05$ ). The unmarked values are not significantly different from 1 ( $P > 0.05$ )

ratios (referred to the unit of tissue N and to the whole liver of a 100 gm. rat) are presented. Whereas calculation of the means and of their standard errors was required for testing the significance of the results (by Fisher's  $t$  test (12)), it was felt that the mean was unduly influenced by the occurrence of a few very high individual values, and that, consequently, the

median described more accurately the set of data obtained in each group or subgroup of experiments. The average changes in the "fat," or in the total fatty acid content, of the liver are also included in Tables II to VI. Production of  $C^{14}O_2$  from labeled fatty acids was significantly higher (Table II) when cystine had been added to the deficient diets, whether or not these diets contained  $\alpha$ -protein or casein, and irrespective of the amounts of fat included in the diet. In some experiments, this effect of dietary cystine

TABLE III

*Comparison of Effects of Various Dietary Supplements on Fatty Acid Oxidation in Liver Homogenates*

Rats on Diets 53, 55, 26, or 47. The substrate was stearate-1- $C^{14}$  or palmitate-1- $C^{14}$

Dietary supplements*	Average change in liver "fat"†	$C^{14}O_2$ ratios‡			
		Per mg liver N		Whole liver§	
		Medians	Means	Medians	Means
	<i>mg per liver§</i>				
Cystine (11)	+945¶	1 91	1 94** $\pm$ 0 31	2 45	2 45** $\pm$ 0 39
Tocopherol (10)	+138	3 61	3 61** $\pm$ 1 12	1 83	3 04** $\pm$ 0 86
Cystine and tocopherol (7)	+713¶	2 03	2 03** $\pm$ 0 15	2 27	2 59** $\pm$ 0 27
Cystine (15)	+362¶	2 59	2 77** $\pm$ 0 32	2 18	2 47** $\pm$ 0 35
Choline (11)	-70	1 51	2 54 $\pm$ 1 01	1 88	2 65 $\pm$ 1 16
Methionine (8)††	-4	1 90	2 74** $\pm$ 0 58	2 06	3 12** $\pm$ 0 93

\*, †, §, ||, ¶, \*\* See corresponding footnotes of Table II

‡ Ratios of  $C^{14}O_2$  produced by the livers of rats on the supplemented diet to the  $C^{14}O_2$  produced, in the same experiments, by the livers of rats on the unsupplemented diet

†† Including two experiments in which, instead of methionine, a mixture of choline and cystine was added to Diet 26

was observed even after only 2 days of supplementation. In Table III are recorded the results of two series of experiments, in which the effects of cystine and of other dietary supplements on the *in vitro* oxidation of fatty acids were compared. Tocopherol alone was as effective as cystine. No further enhancement of fatty acid oxidation was demonstrable in the livers of rats, which had received both cystine and tocopherol supplements. From the medians and the means of the values obtained in the second series of experiments, it would appear that the oxidation of fatty acids *in vitro* was enhanced by choline, methionine, or cystine to approximately the same extent. However, supplementation of the  $\alpha$ -protein diets with choline

was often ineffective, or only slightly effective. Since a number of the rats receiving choline had been on Diet 53 or 55, the means for the whole group are not significantly different from those of the controls (Table III).

Data on the incorporation of  $C^{14}$  in the respiratory  $CO_2$  and in cholesterol of liver slices, incubated with labeled acetate, were inconsistent. Moreover, values for the  $C^{14}$ -fatty acid ratios in the livers of rats maintained on high fat diets were also erratic, perhaps because of the complicating effect of dietary fat in restricting lipogenesis *in vitro* (13). Accordingly these data are not presented here. On the other hand, supplementation

TABLE IV  
*Effects of Supplementing Various Diets with Cystine on  
Fatty Acid Synthesis in Liver Slices*

The substrate was acetate-1- $C^{14}$

Diet No *	Dietary protein	Average change in liver fatty acids†  mg per liver§	$C^{14}$ -Fatty acid ratios†			
			Per mg liver N		Whole liver§	
			Medians	Means	Medians	Means
53 (6)	$\alpha$ -Protein	+363¶	1 81	} 2 80** $\pm$ 0 81	2 85	} 3 69** $\pm$ 1 04
55 (6)	"	+647¶	1 45		1 96	
26 (11)	Casein	-169	4 17	} 2 99** $\pm$ 0 73	3 82	} 3 31** $\pm$ 0 83
47 (4)	"	+447	1 20		1 37	

\* , §, ||, ¶, \*\* See corresponding footnotes of Table II

† Total fatty acid content of the livers of rats on the cystine supplemented diet, less that of the livers of rats on the unsupplemented diet

‡ Ratios of  $C^{14}$ -acetate incorporated in long chain fatty acids of the livers of rats on the supplemented diets to the values obtained, in the same experiment, on the livers of rats on the unsupplemented diet

of any of the four low fat diets with cystine definitely enhanced the synthesis of fatty acids from  $C^{14}$ -acetate (Table IV). Similar enhancements were observed by supplementing Diet 53 or 55 with tocopherol. The medians and the means of the  $C^{14}$ -fatty acid ratios in the livers of rats receiving both cystine and tocopherol were even higher, but, in view of the small number of determinations and of the marked variations in the individual data, the standard errors of the means are quite large (Table V).

In Table VI the effects of high doses of choline chloride, injected terminally, on the oxidation or on the synthesis of fatty acids *in vitro*, have been compared. In line with our previous data (1), following the injections, a marked stimulation of the  $C^{14}O_2$  production was observed in the livers of rats on Diet 26 with added GAA. In some but not in all exper-

TABLE V

*Comparison of Effects of Dietary Supplements of Cystine, Tocopherol, or Cystine and Tocopherol on Fatty Acid Synthesis in Liver Slices*

Rats on Diet 53 or 55 The substrate was acetate-1-C<sup>14</sup>

Dietary supplements*	Average change in liver fatty acid†	C <sup>14</sup> -Fatty acid ratios‡			
		Per mg liver N		Whole liver§	
		Medians	Means	Medians	Means
	mg per liver§				
ystine (12)	+647¶	1 80	2 80** ± 0 81	2 42	2 80** ± 0 51
ocopherol (10)	0	1 55	2 38** ± 0 39	1 86	2 39** ± 0 51
ystine and tocopherol (7)	+732¶	2 15	3 15 ± 1 19	2 69	4 37 ± 1 64

\*, §, ||, ¶, \*\* See corresponding footnotes of Table II

†, ‡ See corresponding footnotes of Table IV

TABLE VI

*Comparison of Effects of Choline Injected Terminally on Fatty Acid Oxidation or Synthesis, in Liver Homogenates or Slices*

All rats on Diet 26 Control rats on Diet 26 with added GAA

Isotopic substrate	Dietary supplement*	Choline injected†	Average change in liver lipides‡	Per mg liver N		Whole liver§	
				Medians	Means	Medians	Means
			mg "fat"§	C <sup>14</sup> O <sub>2</sub> ratios¶			
Stearate	GAA (7)	+	+212	2 46	3 73** ± 0 95	2 34	3 01** ± 0 83
"	Cystine (11)	0	+61	2 42	2 41** ± 0 38	2 32	2 40** ± 0 44
"	" (6)	+	-72	3 54	4 02** ± 0 76	3 47	4 68** ± 1 01
			mg fatty acids§	C <sup>14</sup> Fatty acid ratios¶			
Acetate	GAA (9)	+	+58	1 02	0 89 ± 0 23	0 88	1 01 ± 0 29
"	Cystine (9)	0	-101	4 10	3 11** ± 0 73	3 82	3 60** ± 1 02
"	" (9)	+	+183	1 01	1 66 ± 0 42	1 14	1 77 ± 0 53

\*, §, ||, \*\* See corresponding footnotes of Table II

† 2.5 ml of 0.1 M choline chloride per 100 gm rat in two intramuscular injections 1 hour and 1 hour before the rat was killed

‡ Lipides of the livers of rats injected with choline, or receiving cystine in the diet, less the values obtained, in the same experiment, on the livers of the control rats (not injected with choline and not receiving cystine)

¶ Ratios of the values obtained on the livers of the rats injected with choline, or receiving cystine in the diet, to the values obtained, in the same experiment, on the livers of control rats (not injected with choline and not receiving cystine)

ments fatty acid oxidation in the livers of rats receiving cystine was further enhanced by choline. On the other hand, choline injections did not stimulate lipogenesis in liver slices from rats on the deficient diets, and even seemed to inhibit this process in the livers of rats on the cystine-supplemented diets.

#### DISCUSSION

Since methionine in the rat is a precursor of both cystine and choline, the enhanced fatty acid oxidation in the livers of rats receiving cystine could be ascribed, partly at least, to a sparing action of cystine on the limited amounts of methyl groups available for the synthesis of choline. Such an interpretation would be in line with the effects of choline and cystine on the oxidation of the methyl group of methionine (14).

*In vitro* synthesis of fatty acid from acetate was also enhanced by supplementing the low fat diets with cystine. This finding is in agreement with the earlier demonstration of an increased lipogenesis in intact rats, fed a low casein diet enriched with cystine (15). On the other hand, choline, injected terminally in animals, stimulated the oxidation of fatty acids, but not their synthesis. It seems therefore that choline and cystine act on fatty acid metabolism through different mechanisms and at different biochemical sites.

Whereas, in most of our experiments the so called "antilipotropic effect" of cystine (3) was quite marked, in others the "fat" contents of the livers of animals receiving cystine were similar to, or lower than, those of the rats on the unsupplemented diet. Similar findings with high levels of dietary cystine (above 0.5 per cent) have been noted (16). Moreover, an apparent antilipotropic effect of methionine, added to a threonine-deficient, choline-supplemented diet has been described (17). If an extension of the result obtained on liver preparations to the intact animals is permissible, the occurrence of an antilipotropic action of S-containing amino acids could be ascribed to a stimulation of lipogenesis proportionally greater than that of fatty acid oxidation. At present, since quantitative data are lacking and in view of the number and variety of factors involved, the conditions under which such a disproportion occurs can only be determined empirically.

Our present findings are probably related to some of the metabolic changes which precede the onset of dietary liver necrosis and which are also prevented by either cystine or vitamin E. In slices from the livers of rats fed a 30 per cent *Torula* yeast diet, a rapid respiratory decline, accompanied by a lower incorporation of  $C^{14}$ -acetate into fatty acids, acetoacetate, and  $CO_2$ , was noted. No decrease in  $O_2$  consumption was apparent in homogenates of these livers or in slices from rats with other types of liver damage (18). Livers of rats fed a necrogenic diet (containing 15 per cent  $\alpha$  prote "

and therefore similar to our Diet 55) exhibited lower rates of lipogenesis and a decrease in the levels first of coenzyme A (CoA) and later of diphosphopyridine nucleotide (DPN) (19). The incorporation of cystine-S<sup>35</sup> into liver CoA is also markedly depressed in these animals (20). The role of the low levels of CoA in the development of liver necrosis has been questioned (21). However, since CoA is involved in the synthesis as well as in the oxidation of fatty acids, it is tempting to postulate that, in our experiments, the increased rates of both these processes in the livers of rats receiving cystine might be due primarily to the maintenance of high levels of CoA in the tissue.<sup>2</sup> Indeed, previous observations on pantothenic acid-deficient rats indicate that the CoA levels actually represent a limiting factor for the oxidation of fatty acids (22) and for the synthesis of cholesterol and fatty acids (23) in liver preparations. The similarity in the effects of dietary cystine and tocopherol on both fatty acid oxidation and synthesis *in vitro* could perhaps be explained by the assumption that tocopherol plays a role in the utilization of cystine-S for the synthesis of liver CoA (19).

The excellent technical assistance of Mrs E Hamrick Brady is gratefully acknowledged.

#### SUMMARY

Oxidation of isotopic long chain fatty acids and fatty acid synthesis from C<sup>14</sup>-acetate have been studied in liver homogenates, or slices, from rats previously maintained on various diets low in S-containing amino acids. Both processes are enhanced by supplementing the deficient diets with either cystine or tocopherol. On the other hand, choline, injected terminally in the rats, stimulated oxidation, but not the synthesis of fatty acids in the liver preparations.

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<sup>2</sup> Changes in the amounts and in the intracellular distribution of factors other than CoA have been described in various conditions which lead to fatty or necrotic livers (19, 24, 25). Unquestionably some of these factors, especially DPN, play essential roles in fatty acid metabolism. However, on the basis of the evidence presently available, a specific relationship between the changes observed and the dietary supply of S-containing amino acids is not clearly apparent.

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# ALCOHOL METABOLISM IN "DRINKING" AND "NON-DRINKING" RATS\*

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When rats are allowed a choice between dilute alcohol and water, voluntary alcohol consumption may be used to separate them into several groups, since alcohol consumption is characteristic of the animal. Mardones and coworkers (1) have shown that the amount of alcohol consumed is partly determined by the genetic background of the rats, the number of those classified as "drinkers" having been increased considerably by selective breeding. It was of interest to determine whether differences in the rate or amount of alcohol oxidation could be detected between rats classified as "drinkers" and those classified as "non-drinkers."

## EXPERIMENTAL

Adult male and female rats were used in these studies. Those labeled "Chilean" were either raised in Mardones' laboratory or were bred from the Mardones stock at Harvard University. Adult male and female rats purchased from the Charles River Breeding Laboratories, Cambridge, Massachusetts, were also used. These animals were fed *ad libitum* the diet listed in Table I, and given a choice between 10 per cent alcohol and water. After approximately 60 days, during which time alcohol and water intake were measured daily, the rats were separated into two groups: the drinkers, which had an average intake of 4 to 10 gm of alcohol (absolute) per kilo of body weight per day, and the non-drinkers, which ingested less than 1 gm per kilo per day. After classification, rats from both groups and both strains were injected intraperitoneally with 20 per cent alcohol containing ethanol-1-C<sup>14</sup> at doses which supplied 0.5, 2.0, or 3.0 gm of alcohol per kilo of body weight. After the injection of alcohol, the rat was placed in a respirometer, and the respired CO<sub>2</sub> was collected at hourly intervals for a period of 6 hours. The radioactivity of the CO<sub>2</sub> was used as an index of

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the amount of alcohol metabolized. The method of collecting  $\text{CO}_2$  and the measurement of its activity have been described (3).

### Results

The data in Table II indicate that at none of the three levels of alcohol dosage studied was there any significant difference in the oxidation of the alcohol from either the drinkers or the non-drinkers or from the Chilean and the North American strains of rats. A similar conclusion can be drawn

TABLE I  
*Basal Diet*

Constituents	
	<i>per cent</i>
Casein (purified)	18.3
Sucrose	64.2
Corn oil (Mazola)*	13.8
Salts IV†	3.7
	<i>mg per kg diet</i>
Thiamine chloride	5.00
Riboflavin	6.25
Calcium pantothenate	6.25
Pyridoxine hydrochloride	2.50
Niacin	125.00
<i>p</i> -Aminobenzoic acid	31.25
Inositol	1.25
Biotin	1.25
Folic acid	2.50
Choline chloride	250.00

\* Fortified with 0.5 gm of cod liver oil and 7.5 mg of  $\alpha$ -tocopherol acetate

† Hegsted *et al* (2)

from the cumulative recoveries of  $\text{C}^{14}\text{O}_2$ , plotted for two different alcohol dosages (Figs 1 and 2). These recoveries from the rats receiving 2 gm of alcohol per kilo give a nearly linear curve during the first 6 hours. The slope of such a line indicates an oxidation of 14.7 per cent of the dose per hour, or a mean value of 294 mg per kilo per hour. A closer inspection of the points suggests that, in conformity with previous work (3), the curve is actually sigmoid, being below the line during the first hours and above the line at 4 and 5 hours. Plotting the cumulative recoveries from groups of animals tends to "smooth out the curve" and make the changes in oxidation in relation to alcohol level only slightly evident. The curve for the rats which received only 0.5 gm per kilo decreased rapidly with time, and during the 2nd hour 29.1 per cent of the dose, *i.e.* 196 mg per kilo per hour.

was oxidized This value is significantly lower than the rate at the higher dosage

TABLE II

*Metabolism of Ethanol-1-C<sup>14</sup> by Two Strains of Alcoholic and Non-Alcoholic Rats*

Dose of 20 per cent ethanol 1 C <sup>14</sup>  gm per kg	Chilean strain				North American strain			
	No of rats	Drinkers, per cent metabolized*†	No of rats	Non-drinkers, per cent metabolized	No of rats	Drinkers, Per cent metabolized	No of rats	Non-drinkers, per cent metabolized
0.5	4	93.3 ± 4.0	4	93.7 ± 2.2	3	85.5 ± 1.85	3	88.7 ± 4.2
2.0	4	78.5 ± 4.4	6	74.0 ± 1.7	5	78.4 ± 4.4	5	74.1 ± 2.1
3.0					10	44.0 ± 2.6	5	39.0 ± 0.9

\* Drinkers ingested 4 to 10 gm of alcohol (absolute) per kilo of body weight per day over a 60 day period, non-drinkers ingested less than 1 gm of alcohol per kilo of body weight over a 60 day period

† In 6 hours

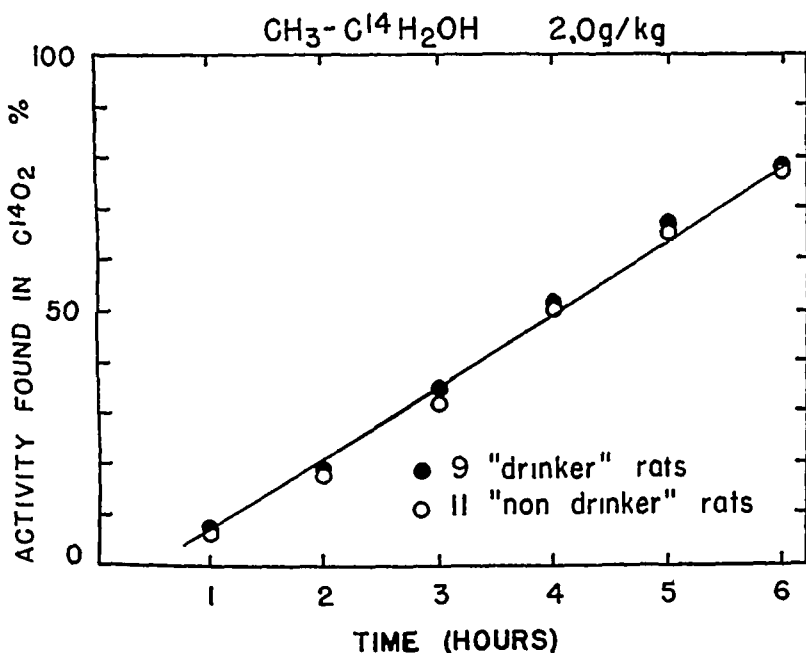


FIG 1 Cumulative recoveries of activity in rats given 2 gm of alcohol per kilo of body weight

Although the average recoveries of C<sup>14</sup>O<sub>2</sub> from the Chilean rats at the 0.5 gm dose were somewhat higher than the values obtained with the North American strain (Table II), these differences are not statistically

significant. Thus, it appears unlikely that there is a strain difference in the ability to oxidize alcohol.

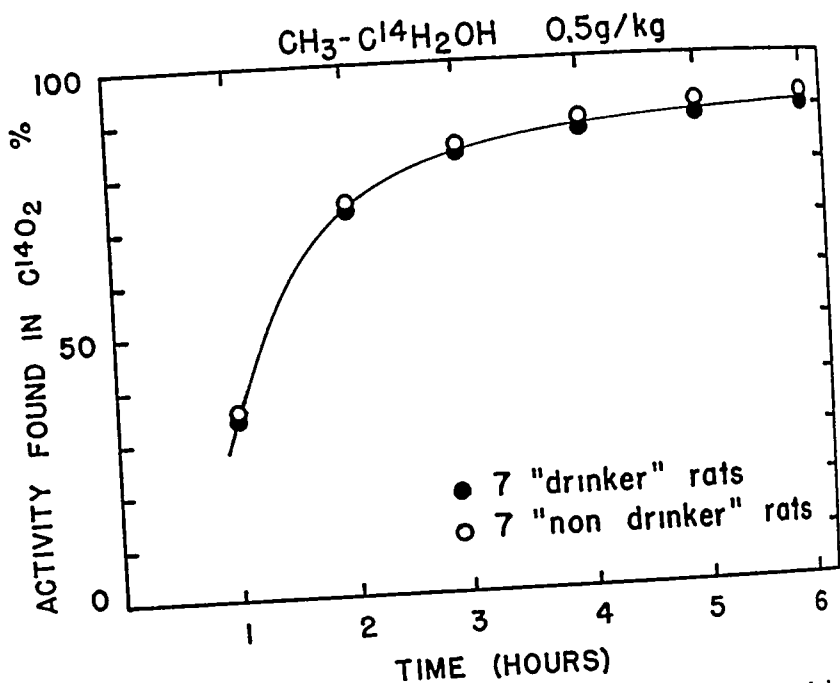


FIG. 2 Cumulative recoveries of animals given 0.5 gm of alcohol per kilo of body weight

#### DISCUSSION

Previous studies (3) demonstrated a dependence of the rate of alcohol oxidation upon the alcohol dose administered. Maximal oxidation of 30 to 400 mg per kilo of body weight per hour was found to occur at levels of 2.0 to 2.5 gm per kilo of body weight, values which approximate the found in the present study when 2.0 gm of alcohol were administered. The rats selected as drinkers in this study were consuming voluntarily from 4 to 10 gm of alcohol per kilo of body weight per day. Thus, the total alcohol consumption per day, particularly of those consuming the large amounts, approximated the maximal capacity of the animal to oxidize alcohol during the entire day. Apparently the ability to metabolize alcohol limits the alcohol intake, since at higher levels of consumption the alcohol would accumulate in the body and the animals would become intoxicated.

The observed difference in the rate of alcohol oxidation at different doses of alcohol is in agreement with the results reported by Vitale *et al* (1) and with the course of the decrease of the blood level in dogs reported by Marshall and Fritz (4). As may be appreciated from an inspection of

Fig 2, changes in the rate of alcohol oxidation with changes in blood concentration cannot be readily demonstrated at high levels of alcohol dosage

The results obtained indicate that the different drinking behaviors of rats classified as drinkers and non-drinkers seem not to be a consequence of a difference in the ability to oxidize alcohol. Furthermore, the prolonged ingestion of alcohol by the drinker rats seems not to increase their ability to oxidize alcohol. Thus, if some "adaptation" to alcohol ingestion may occur, it does not seem to consist of an increase in the enzymes necessary for alcohol combustion.

Although no difference in the ability to oxidize alcohol in drinkers and non-drinkers was demonstrable in these studies, it may not be correct to conclude that there are no differences in metabolism of alcohol in these animals. We have measured only the amount consumed by the main oxidative pathways for alcohol. Undoubtedly, a small amount is disposed of by incorporation into other body constituents and metabolites. Whether these mechanisms, which account for only a small fraction of the total oxidized, may have an effect on the appetite for alcohol is problematical. The fact that the rats are "different" presupposes differences in metabolism, although not necessarily in alcohol metabolism. Studies upon acetate and pyruvate metabolism have been initiated, which will be reported in a later paper.

#### SUMMARY

The rates of alcohol oxidation in rats which have a high voluntary alcohol consumption have been compared with those which refuse alcohol offered to them. No differences in the amount of alcohol oxidized at three different doses of alcohol were found.

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# $O^{18}$ AND $P^{32}$ EXCHANGE REACTIONS OF MITOCHONDRIA IN RELATION TO OXIDATIVE PHOSPHORYLATION\*

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The study of oxidative phosphorylation<sup>1</sup> by the classical techniques of fractionation and isolation of the purified reactants has not been feasible because of the lability of the reaction systems. Consequently indirect approaches to the problem have been adopted. This paper reports results from one such approach, namely, study by the use of  $P^{32}$  and  $O^{18}$  of enzymic exchange reactions of phosphate oxygen and of inorganic orthophosphate ( $P_i$ )<sup>2</sup> which appear to be intimately related to oxidative phosphorylation.

Preliminary reports from this laboratory have described the finding that rat liver mitochondria will catalyze a rapid exchange of  $P_i$  with the phosphate of adenosine triphosphate (ATP) ( $P_i$ -ATP exchange) as well as the exchange of phosphate oxygens with those of water ( $O_{P_i}$ - $H_2O$  exchange) in the absence of added substrate and oxygen uptake (1). Swanson has recently reported a similar exchange of  $P_i$  with ATP which was catalyzed by mitochondria (2). The occurrence of a rapid exchange of phosphate oxygen in systems which catalyze net oxidative phosphorylation has been previously demonstrated by Cohn (3) and further studied by Cohn and Drysdale (4). A reversible incorporation of  $P_i^{32}$  into ATP catalyzed by brain homogenates and dependent upon net oxygen uptake has been

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<sup>1</sup> The term oxidative phosphorylation, as used herein, connotes the conversion of inorganic orthophosphate to ester or anhydrophosphate compounds, coupled to enzymic oxidations other than the reactions of substrates with oxidized pyridine nucleotides.

<sup>2</sup> The abbreviations used are  $P_i$ , inorganic orthophosphate, ATP, adenosine triphosphate, ADP, adenosine diphosphate, ATPase, adenosinetriphosphatase, AMP, adenosine monophosphate, EDTA, ethylenediaminetetraacetic acid, DNP, 2,4-dinitrophenol.

shown by Lee and Eiler (5), and Ernster, Ljunggren, and Lindberg demonstrated that mitochondria, when oxidizing  $\alpha$ -ketoglutarate, catalyze turnover of phosphate in ATP at a rate which exceeds the rate of phosphate uptake (6)

This paper gives further details of experiments which demonstrate the  $P_i$ -ATP and  $O_{P_i}$ - $H_2O$  exchanges, the effect of various experimental conditions on these exchanges, and some interpretation of the results

### *Materials and Methods*

Mitochondria from livers of fasted rats were prepared essentially by the technique of Schneider (7) as described previously (8), by using 0.25 M sucrose containing  $10^{-4}$  M ethylenediaminetetraacetic acid (EDTA) with or without 0.005 M  $\beta$ -glycerophosphate at pH 7.2. The mitochondria were suspended in a volume (in ml) of the sucrose solution equivalent to the number of gm of fresh liver.

Reactions were stopped by addition of appropriate volumes of trichloroacetic or perchloric acid solution, and analyses for  $P_i$ ,  $P^{32}$ , and  $O^{18}$  were made essentially as described previously (8, 9). Absorption of  $P_i$  on the charcoal, used to remove nucleotides from solutions for determination of radioactivity, was minimized by preliminary treatment of the charcoal with a 5 per cent (w/v) trichloroacetic acid solution containing 0.05 M  $P_i$ . Other experimental details are given with Tables I to VI and Fig. 1 to 4.

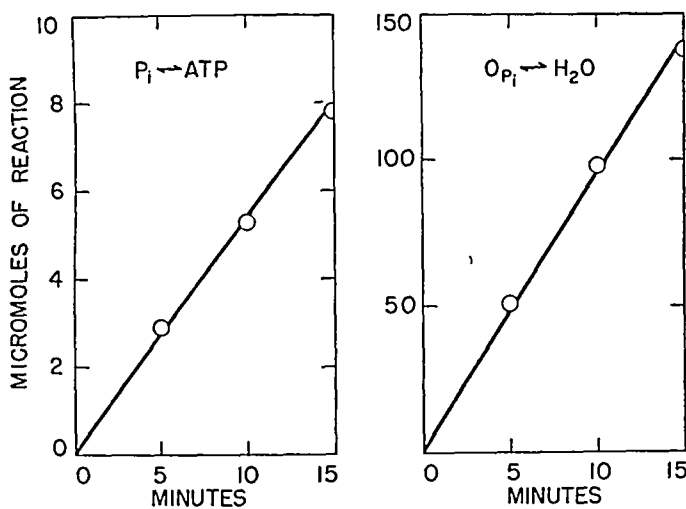
### *Results*

*Time Course of Exchange Reactions*—Liver mitochondria isolated from rats fasted for 24 hours and incubated without added substrate will catalyze an extensive exchange of  $P_i^{32}$  with the phosphate of ATP, and a much more rapid exchange of phosphate oxygen with water oxygen (1). The time course of these exchange reactions observed in a typical experiment is shown in Fig. 1. The ordinates give values for the total amount of the exchange reactions. These values were calculated from the expected logarithmic approach to isotopic equilibrium with the use of the following relationship (10): the extent of reaction =  $\ln(1 - F) \times (-AB)/(A + B)$ , where  $A$  and  $B$  are the amounts of the reactants undergoing exchange and  $F$  is the fraction of isotopic equilibrium attained. The results show that both the  $P_i$ -ATP and the  $O_{P_i}$ - $H_2O$  exchange reactions continue in a nearly linear manner over the 15 minute reaction period. For each phosphate exchange between  $P_i$  and ATP, 18 phosphate oxygen atoms had exchanged. In seven other similar experiments with 10 minute incubation times, the ratio of oxygen atoms to phosphate exchange has ranged from 14.7 to 18.4.

with an average of 16.7. No  $O_2$  uptake was detectable after the 5 minute preincubation period (1)

The  $P^{32}$  exchange appears to be specific for adenine nucleotides. When the ATP was replaced by inosine triphosphate or uridine triphosphate, slight or no incorporation of  $P^{32}$  into nucleotides was found in a 20 minute incubation period.

*Inhibition of Exchange Reactions by 2,4-Dinitrophenol—Low concentra-*



TIME COURSE OF THE  $P_i \rightarrow ATP$  AND  $O_{P_i} \rightarrow H_2O$  EXCHANGES

Fig. 1 The time course of  $P_i$ -ATP and  $O_{P_i}$ - $H_2O$  exchange reactions. 1 ml of washed rat liver mitochondria in 0.25 M sucrose,  $10^{-4}$  M EDTA, and 0.005 M  $\beta$ -glycerophosphate, pH 7.2, was added to a 3 ml solution containing 37.5  $\mu$ moles of ATP, pH 7.4, 37.5  $\mu$ moles of  $MgSO_4$ , and 265  $\mu$ moles of KCl. The mixture was incubated for 5 minutes at 28°, after which 1 ml of a solution containing potassium phosphate buffer labeled with  $P^{32}$  and  $O^{18}$ , pH 7.3, and KCl (to osmolarity = 0.25) was added. The incubation was continued, and the reaction was terminated at the appropriate time by the addition of 1.5 ml of 30 per cent trichloroacetic acid solution.  $P^{32}$  present in the ATP and  $O^{18}$  present in the  $P_i$  were determined on appropriate aliquots.

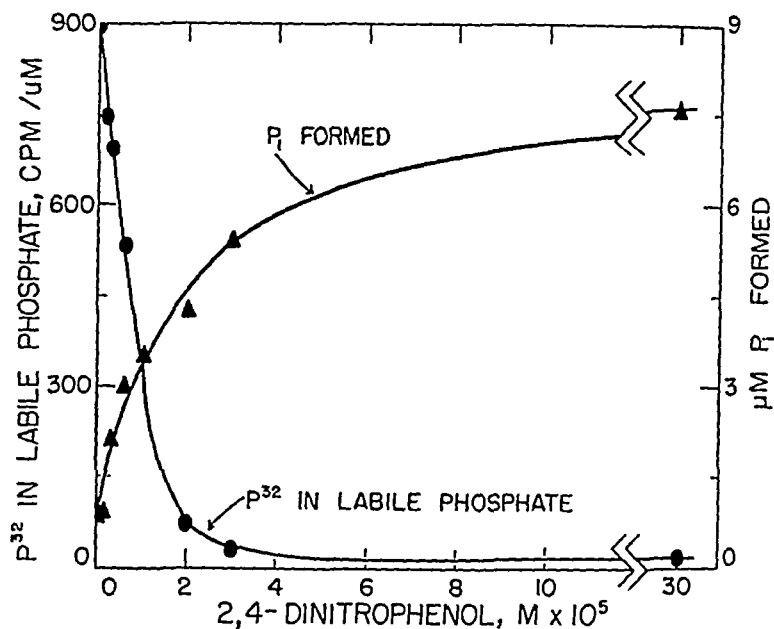
tions of 2,4-dinitrophenol markedly inhibit the  $P_i$ -ATP exchange. Fig. 2 shows the effect of increasing concentrations of 2,4-dinitrophenol on the  $P_i$ -ATP exchange as well as on the acceleration of ATP cleavage by mitochondria. Under the same conditions employed for the experiments reported, 2,4-dinitrophenol also markedly inhibited the  $O_{P_i}$ - $H_2O$  exchange, as would be anticipated from the earlier observations of Cohn (3). Both exchange reactions are inhibited by azide (8).

Additions of adenosine monophosphate (AMP) and, to a lesser extent, of adenosine diphosphate (ADP) also inhibit the  $P_i$ -ATP and  $O_{P_i}$ - $H_2O$



exchange reactions<sup>3</sup> The effects of 2,4-dinitrophenol may thus in part reflect increases in the concentrations of ADP and AMP in the medium

*Possible Participation of Mitochondrial "ATPase" in  $P_i$ -ATP Exchange*—Considerations of the energy involved make unlikely the possibility that the  $P_i$ -ATP exchange reaction results from a reversal of ATP hydrolysis. Furthermore, Koshland *et al* (11) demonstrated that incorporation of  $P_i$



RELATIVE EFFECT OF DINITROPHENOL ON THE  $P_i^{32}$ -ATP REACTION AND ATP CLEAVAGE

FIG. 2 The effect of DNP on the  $P_i$ -ATP reaction and ATP cleavage. A solution containing 15.4 μmoles of potassium phosphate, pH 7.4, labeled with  $P_i^{32}$ , and an appropriate amount of DNP was added to an incubated solution (4 minutes) containing 7.4 μmoles of ATP, pH 7.4, 7.5 μmoles of  $MgCl_2$ , 50 μmoles of KCl, and 0.2 ml of a rat liver mitochondrial suspension in 0.25 M sucrose and  $10^{-4}$  M EDTA. The final volume was 1 ml. The mixture was incubated for 10 minutes at 30°, and the reaction was terminated by addition of 5 per cent trichloroacetic acid.

into ATP during hydrolysis of ATP by actomyosin preparations was extremely small. As an additional test, crude mitochondrial ATPase was prepared as described by Kielley and Kielley (12). With such preparations, no incorporation of  $P_i^{32}$  into ATP was detected under such conditions that the amount of incorporation with intact mitochondria of comparable ATPase activity would have been very marked.

Another possibility is that the mitochondrial ATPase might, in inter-

<sup>3</sup> Luchsinger, W. W., and Boyer, P. D., unpublished experiments.

mitochondria, catalyze a transfer of phosphate from ATP to an acceptor other than water by means of an enzyme phosphate intermediate. The marked inhibition of the ATPase by adenosine diphosphate (13) could conceivably result from reaction of an enzyme phosphate intermediate with the ADP to form ATP. To test such a possibility, measurements were made of the incorporation of ADP-P<sup>32</sup> into ATP when the ATPase was inhibited by added ADP. Some incorporation of ADP-P<sup>32</sup> was found, however, this was only slightly decreased by addition of sufficient *p*-mer-

TABLE I

*Phosphate Oxygen Exchange Accompanying Net Oxidative Phosphorylation*

The 20 ml reaction mixture in a 125 ml Erlenmeyer flask contained 3 ml of 0.3 M potassium phosphate O<sup>18</sup> at pH 7.4, 1.5 ml of 0.02 M potassium AMP at pH 7.2, 2 ml of 0.075 M MgSO<sub>4</sub>, 2 ml of 2 × 10<sup>-4</sup> M cytochrome *c*, 4 ml of 0.1 M potassium glutamate, 3 ml of 0.25 M sucrose containing 10<sup>-4</sup> M EDTA, 2 ml of 0.2 M glucose containing 35 mg of Pabst crude hexokinase, and 2.0 ml of a washed liver mitochondrial suspension. Shortly after addition of the mitochondria, the flask was placed in a bath at 37°, 2.0 ml removed for "zero time" P<sub>i</sub> and P<sub>i</sub>-O<sup>18</sup> determinations, two 3.0 ml aliquots placed in Warburg flasks for oxygen uptake measurements, and these and the 125 ml Erlenmeyer flask shaken for 15 minutes. The reaction was stopped by addition of 0.2 ml of 30 per cent trichloroacetic acid per ml of reaction mixture. Nucleotides were removed by charcoal absorption, and O<sup>18</sup> determinations were made as described in the text.

O <sub>2</sub> uptake per ml	P <sub>i</sub> uptake per ml	Atom per cent excess O <sup>18</sup>		
		Initial P <sub>i</sub>	Final P <sub>i</sub>	Phosphate of glucose-6-phosphate*
<i>microatoms</i>	<i>μmoles</i>			
2.5	6.0	0.392	0.310	0.159

\* The value is for the three oxygen atoms that were transferred with the phosphate group to form glucose-6-phosphate.

curibenzoate to inhibit the ATPase completely. The maximal amount of incorporation attributable to the ATPase was small and much less than that expected if the inhibition of ATP breakdown resulted from reaction of ADP with a phosphorylated intermediate.

*Phosphate Oxygen Exchange Accompanying Net Oxidative Phosphorylation*  
—The data in Table I show the extent of exchange of the oxygens of phosphate taken up and transferred to glucose in net oxidative phosphorylation with glutamate as a substrate. The decrease in the excess O<sup>18</sup> of the P<sub>i</sub> during the incubation shows the expected extensive exchange of the oxygen of P<sub>i</sub>. However, the oxygen atoms transferred with the phosphate to form glucose-6-phosphate had considerably less excess O<sup>18</sup> than the P<sub>i</sub> of the reaction medium, thus the ester phosphate was not equivalent in O<sup>18</sup>

content to the  $P_i$  from which it was derived. If the additional oxygen exchange occurred by exchange reactions of  $P_i$ , localized at the site of phosphorylation, and thus not in equilibrium with the  $P_i$  of the medium, the  $O^{18}$  loss may be estimated to be equivalent to exchange of 3.1 oxygen atoms per phosphate eventually appearing as glucose-6-phosphate.<sup>4</sup>

In experiments similar to that outlined in Table I, but with adenosine monophosphate as a phosphate acceptor, the phosphate transferred to the AMP had less  $O^{18}$  content than the  $P_i$  of the reaction medium. The additional phosphate oxygen exchange in two experiments with different mit-

TABLE II

*Effect of Aging Mitochondria on Exchange Reactions*

1 ml of mitochondria, prepared in 0.25 M sucrose,  $10^{-4}$  M EDTA, and 0.005 M glycerophosphate at pH 7.2 and aged as indicated, was added to 3 ml of a solution containing 37.5  $\mu$ moles of ATP at pH 7.4, 37.5  $\mu$ moles of  $MgCl_2$ , and 253  $\mu$ moles of KCl and was incubated at 30°. After 5 minutes, 1 ml of a solution containing 10  $\mu$ moles of potassium phosphate labeled with  $P^{32}$  and  $O^{18}$  and 26  $\mu$ moles of KCl was added. The incubation was continued for an additional 10 minute period.

Aged at 37°	$\Delta P_i$	Atom per cent excess $O^{18}$ of $P_i$ *		Total radioactivity in nucleotides†
		Observed	Resulting from O exchange†	
min	$\mu$ moles			c.p.m.
0	4.0	0.171	0.199	15,400
0	2.0	0.182	0.198	15,800
15	8.5	0.172	0.178	11,700
30	29.5	0.239	0.040	1,610
60	29.5	0.249	0.030	280
90	39.5	0.241	0.014	330

\* The atom per cent excess  $O^{18}$  in  $P_i$  of the zero time control was 0.390.

† Decrease in atom per cent excess  $O^{18}$  over that caused by the  $P_i$  formed during the incubation.

‡ Total radioactivity present in the initial  $P_i$  was equivalent to 102,000 c.p.m.

ochondrial preparations was equivalent to exchange of 1.6 and 3.1 oxygen atoms per  $P_i$  eventually taken up and transferred to AMP.

*Effect of Aging Mitochondria on Exchange Reactions*—Incubation of the mitochondria in 0.25 M sucrose at 37° resulted in a marked decrease in the

<sup>4</sup> This calculation is based on the use of the average  $O^{18}$  concentration of the  $P_i$  of the medium (0.351 atom per cent excess) and the relation: microatoms of O exchanged per micromole of phosphate =  $9.2 \log E_0/E$  in which  $E_0$  and  $E$  are the initial and final excess  $O^{18}$  concentrations of the phosphate undergoing exchange. The estimated number of oxygen exchanges would be three-quarters of the calculated value, only the 3 oxygen atoms transferred with the phosphate group to an intermediate were considered to participate in the exchange.

catalysis of the  $O_{P_i}$ - $H_2O$  and the  $P_i$ -ATP exchange reactions, as well as the expected increase in the rate of ATP hydrolysis. The susceptibility of mitochondria to the effects of aging varied with different preparations, results of a typical experiment are given in Table II. In this experiment aging for 15 minutes had little effect and aging for 30 minutes a marked effect on the extent of the exchange reactions. The effect on the oxygen exchange reaction roughly paralleled the increase in the extent of ATP hydrolysis. The effect of aging on the exchange reactions may be explained in part by the accumulation of ADP and AMP, which markedly inhibit the exchange reactions.<sup>3</sup> Aged mitochondria also show a decreased capacity for net oxidative phosphorylation, and this decrease may be partially restored by ATP addition (14).

*Effect of Various Additions and of Anaerobic Conditions upon Exchange Reactions*—Table III shows the effects of various additions on the amount of the  $P^{32}$  and  $O^{18}$  exchange. Of particular interest is the small or negligible effect on the exchange reactions of anaerobic conditions or of  $10^{-4}$  M cyanide, with or without succinate present. With oxygen uptake blocked by cyanide or anaerobic conditions and with substrate present, the electron carriers should be nearly completely reduced (15). The small decrease in the exchange produced by evacuation of and admission of air probably reflects damage to mitochondria which results from slight frothing during the evacuation. The effect of an increasing concentration of cyanide on the amount of exchange between  $P_i^{32}$  and ATP is shown in Fig. 3. Cyanide at  $10^{-4}$  M, sufficient for nearly complete inhibition of cytochrome oxidase, had little effect on the exchange rates. Higher concentrations of cyanide produced some inhibition. However, the inhibition did not increase with increase in cyanide concentration in the manner that would be anticipated if only one reactant were affected. The results suggest that at least two distinct pathways exist for the exchange, one of which is cyanide-sensitive while the other is relatively insensitive to cyanide inhibition.

*Requirement of ATP for Oxygen Exchange Reaction*—The data in Table IV illustrate the dependence of the  $P_i$  oxygen exchange reaction upon the addition of ATP when mitochondria are incubated in the presence of low 2,4-dinitrophenol concentrations. Calculations of the amount of the exchange reactions in the presence of the 2,4-dinitrophenol are subject to possible error because of the relatively rapid formation of  $P_i$  from ATP, the data are thus reported only as atom per cent excess  $O^{18}$  and total radioactivity of the nucleotides. The first three experiments are controls for zero time and for incubation without 2,4-dinitrophenol added, the exchanges proceeded in the expected manner. When 2,4-dinitrophenol was present and ATP was not added, the oxygen exchange reaction was abol-

ished within experimental error. The presence of ATP, added either before or 5 minutes after the mitochondria, partially restored both the  $O_2$ ,  $H_2O$  and  $P_i$ -ATP exchange reactions.

TABLE III  
*Effect of Reaction Conditions on  
Exchange Reactions*

The reaction tubes contained, per ml, 7.5  $\mu$ moles of ATP, 7.5  $\mu$ moles of  $MgCl_2$ , 15  $\mu$ moles of  $P_i$ , 0.2 ml of rat liver mitochondria in 0.25 M sucrose,  $10^{-4}$  M EDTA, 0.005 M  $\beta$ -glycerophosphate, pH 7.2, and sufficient KCl to make the final osmolarity equal to 0.25, plus additions as indicated. The reaction mixtures, which lacked the  $P_i^{32}O^{18}$ , were preincubated 5 minutes, at which time the  $P_i^{32}O^{18}$  was added. The completed samples were incubated for 10 minutes at  $30^\circ$  and pH 7.2 to 7.4, after which an equal volume of 5 per cent trichloroacetic acid was added to terminate the reaction. The  $P^{32}$  present in the nucleotides and the  $O^{18}$  in the  $P_i$  were determined on appropriate aliquots.

Conditions	Specific radioactivity in nucleotides as per cent of aerobic control	Decrease in atom per cent excess $O^{18}$ in $P_i$ due to exchange
Aerobic control	100	0.267*
KCN, 0.1 $\mu$ mole per ml	103	
" 0.1 " " "	109	0.230
" 0.1 " " " sodium succinate, 10 $\mu$ moles per ml	102	
KCN, 0.1 $\mu$ mole per ml, sodium succinate, 10 $\mu$ moles per ml	97	0.279
Sodium citrate, 1 $\mu$ mole per ml	160	
" pyrophosphate, 1 $\mu$ mole per ml	73	
" phosphite, 10 $\mu$ moles per ml	89	
" " 34 " " "	56	
" phosphate, 34 " " " †	60	
Sucrose, 700 $\mu$ moles per ml	0	
p-Mercuribenzoate, 0.1 $\mu$ mole per ml	28	
Aerobic control	100	
Anaerobic control (evacuated, air readmitted)	82	0.607‡
"	83	0.587
" sodium succinate, 10 $\mu$ moles per ml	85	0.684

\* The atom per cent excess  $O^{18}$  in  $P_i$  of zero time control was 0.412.

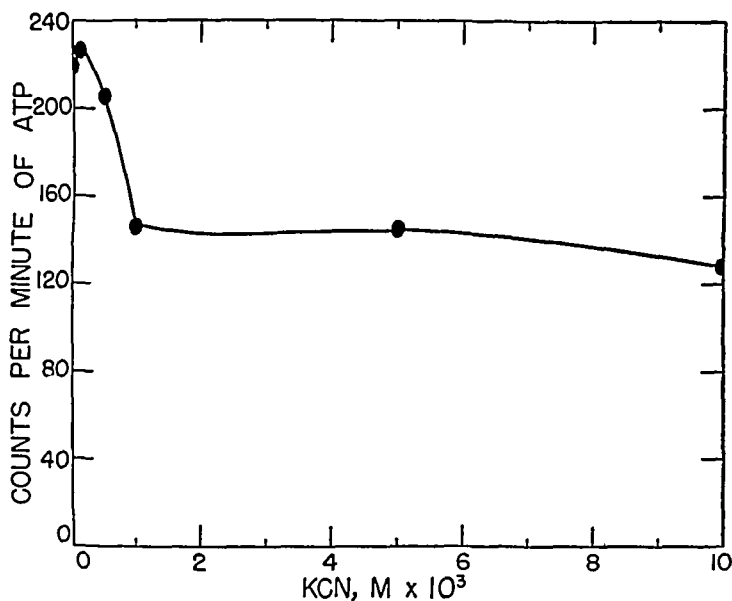
† In excess of the normal amount present, 15  $\mu$ moles per ml.

‡ The atom per cent excess  $O^{18}$  in  $P_i$  of zero time control was 1.19.

According to the data in Table V, the oxygen exchange continues in the absence of added ATP if 2,4-dinitrophenol is not present. By aging for short periods, the exchange occurring in the absence of added ATP can be

abolished and subsequently partially restored by the addition of adenosine triphosphate

The mitochondria incubated without substrate and ATP, but in the presence of 2,4-dinitrophenol, would be expected to be depleted of their supplies of "high energy" phosphate compounds. These results thus give further evidence for the dependence of the  $P_i$  oxygen exchange reaction on a source of high energy phosphate



### EFFECT OF KCN ON THE $P_i^{32}$ -ATP EXCHANGE

Fig 3 Effect of KCN on the  $P_i^{32}$ -ATP exchange. Potassium phosphate (10.9  $\mu$ moles) labeled with  $P^{32}$  was added to a mixture (incubated for 4 minutes) which contained 5  $\mu$ moles of ATP, 6.8  $\mu$ moles of  $MgCl_2$ , varying amounts of KCN, and 0.2 ml of rat liver mitochondria in 0.25 M sucrose and  $10^{-4}$  M EDTA. The sample (final volume 1 ml) was incubated for an additional 10 minutes at  $26^\circ$ , and the reaction was terminated by addition of 1 ml of 5 per cent perchloric acid. The  $P^{32}$  present in the ATP was determined on a suitable aliquot.

*Possible Catalysis of Oxygen Exchange by Molybdoflavoprotein*—The reduction of cytochrome *c* by aldehydes in the presence of aldehyde oxidase has been shown to require the addition of  $P_i$  and  $MoO_3$  (16). The requirement for  $P_i$  might conceivably reflect the transient formation of an unstable phosphate compound which was cleaved by water but which, in the intact cell, could act as a donor of high energy phosphate. This possibility was checked in an experiment in which 11.7  $\mu$ moles of cytochrome *c* were reduced in the presence of 50  $\mu$ moles of  $P_i$  and of  $H_2O$  containing 1.0

atom per cent excess  $O^{18}$ .<sup>5</sup> No incorporation of water oxygen into the  $P_i$  was detected

*Phosphate Oxygen Exchange in Intact Organisms*—If the rapid  $O_{P_i}$ - $H_2O$  exchange occurs in intact animals,  $O^{18}$  administered as  $H_2O$  should appear in the inorganic phosphate and other phosphate compounds. Phosphate compounds may also derive oxygen from substrates in various coupled

TABLE IV  
*Requirement of ATP for Oxygen Exchange Reaction  
in Presence of 2,4-Dinitrophenol*

The reaction was initiated by adding 1 ml of washed rat liver mitochondria (0.25 M sucrose,  $10^{-4}$  M EDTA, and 0.005 M  $\beta$ -glycerophosphate at pH 7.2) to a 4.5 ml mixture at pH 7.3 containing 37.5  $\mu$ moles of  $MgCl_2$ , 75  $\mu$ moles of potassium phosphate labeled with  $P^{32}$  and  $O^{18}$ , KCl sufficient to make a final osmolarity of 0.25, and the amounts of 2,4-dinitrophenol and ATP as indicated. Incubation was at 30°, and the reaction was terminated by addition of trichloroacetic acid.

2,4-Dinitrophenol	ATP	Time	$\Delta P_i$	Atom per cent excess $O^{18}$ in $P_i$		Total radioactivity nucleotides <sup>†</sup>
				Observed	$\Delta$ resulting from O exchange*	
$M \times 10^3$	$\mu$ moles	min	$\mu$ moles			c.p.m.
0	37.5	0	0	0.345	0	0
0	37.5	5	2.2	0.166	0.170	7470
0	37.5	10	5.5	0.071	0.251	9680
2.7	0	5		0.343	0.002	28
2.7	0	10		0.349	-0.004	28
2.7	37.5	5	14.4	0.220	0.070	1130
2.7	37.5	10	27.5	0.170	0.083	1260
2.7	37.5 added at 5 min	10	16.5	0.239	0.044	873

\* Decrease in atom per cent excess  $O^{18}$  over that resulting from  $\Delta P_i$  during incubation

† The total radioactivity present in the  $P_i$  added was 39,200 c.p.m.

reactions (17). However, such exchange would be expected to be small compared to the exchange with water. According to these expectations, the acid-extractable inorganic phosphate and labile organic phosphate fractions of the liver and muscle from a rat which weighed 240 gm, which had imbibed approximately 17 gm of  $O^{18}$  water (1.0 atom per cent ex-

<sup>5</sup> This experiment was made possible through the cooperation of Dr. Fred Crane and Dr. Joseph Glenn, Institute for Enzyme Research, University of Wisconsin, who kindly carried out the cytochrome reduction with aldehyde oxidase purified at the Enzyme Institute.

O<sup>18</sup>) over a 6 hour period, contained from 0.06 to 0.09 atom per cent excess O<sup>18</sup>

Exchange of phosphate oxygens with those of water does not occur in phosphate transfer reactions associated with glycolysis (3, 9, 18). Thus, if the O<sub>P<sub>i</sub></sub>-H<sub>2</sub>O exchange found with rat liver mitochondria and intact rats results from reactions associated with oxidative phosphorylation, then such exchange might be expected to be absent in an organism which lacks the

TABLE V  
*Requirement of Added ATP for Exchange Reactions  
with Aged Mitochondria*

1 ml of mitochondrial suspension, prepared as previously described and aged as indicated, was added to 3 ml of a solution containing 37.5  $\mu$ moles of ATP, 37.5  $\mu$ moles of MgCl<sub>2</sub>, and 253  $\mu$ moles of KCl at pH 7.4 and incubated at 30°. After 5 minutes 1 ml of potassium phosphate solution (pH = 7.3) containing 75  $\mu$ moles of phosphate labeled with P<sup>32</sup> and O<sup>18</sup> and 26  $\mu$ moles of KCl was added, and the incubation was continued for an additional 10 minutes

Experiment No	Mitochondria aged at 37°	Conditions	$\Delta P_i$	Atom per cent excess O <sup>18</sup> in P <sub>i</sub> *		Total radioactivity in nucleotides†
				Observed	Decrease resulting from O exchange‡	
	min		$\mu$ moles			c p m
1	0	Complete system	3.9	0.724	0.391	11,500
	0	No ATP		0.904	0.269	65
2	0	Complete system	5.2	0.128	0.231	7,130
	15	" "	11.0	0.199	0.135	4,600
	15	No ATP		0.240	0.125	77
	30	Complete system	22.5	0.176	0.119	2,230
	30	No ATP		0.372	0.005	0

\* The atom per cent excess O<sup>18</sup> of P<sub>i</sub> in zero time control was 1.173 for Experiment 1 and 0.383 for Experiment 2

† This represents a decrease in atom per cent excess O<sup>18</sup> over that caused by  $\Delta P_i$  during incubation

‡ The total radioactivity of the P<sub>i</sub> added was 39,400 c p m

requisite enzymes for oxidative phosphorylation. As a test of this possibility, measurement was made of the phosphate oxygen exchange with a microorganism, *Leuconostoc mesenteroides* P-60, which is incapable of oxygen uptake and which derives its energy from glucose breakdown to lactic acid, ethanol, and CO<sub>2</sub>.

To find out whether exchange occurred between P<sub>i</sub> oxygen and water oxygen, cells were grown in a medium containing H<sub>2</sub>O<sup>18</sup> and were harvested, and the O<sup>18</sup> of the trichloroacetic acid-extractable phosphate was deter-



mined. The results of such an experiment (Table VI) show that little or no exchange of the oxygen of the cellular phosphate with oxygen of water had occurred.

### DISCUSSION

The continued net formation of ATP from  $P_i$  and ADP coupled to electron transport must involve loss of an oxygen from the  $P_i$  or ADP to form water either directly or through one or more intermediates. The reversal of the formation of ATP by such reactions could result in the incorporation of water oxygen into the  $P_i$  or ADP. Present information definitely points to the explanation that the rapid exchange of  $P_i$  oxygen with water oxygen as described herein results from the reversal of one, or more than one, of the phosphate uptake reactions of oxidative phosphorylation. Based on the validity of this explanation, the conclusion may be reached that the phosphate uptake reaction or reactions concerned are

TABLE VI  
*Lack of Exchange of Phosphate Oxygen  
with  $H_2O$  in *L. mesenteroides* P-60*

Experiment No	Atom per cent excess $O^{18}$	
	$H_2O$	Phosphate
1	1.19	<0.10
2	1.19	<0.05

a dynamic state in both the presence and the absence of net oxidative phosphorylation. In addition, the following characteristics of the process of the oxidative phosphorylation concerned would appear highly likely: (a) the formation of ATP does not occur by a direct reaction of  $P_i$  with ADP but involves at least two steps, (b) the first step, which is responsible for the phosphate oxygen exchange reaction, involves direct or eventual formation from either ADP or  $P_i$  of an intermediate which does not contain all the oxygens associated with the initial ADP or  $P_i$ ,<sup>6</sup> and (c) the oxygen exchange reaction is not coupled directly to oxidation and reduction of electron carriers. Therefore, electron carrier oxidation results in the formation

<sup>6</sup> This is an important distinction from alternative suggestions in which the immediate precursor to ATP would retain all the oxygens associated with the original  $P_i$  or ADP; direct formation of ATP from such a precursor would necessitate that the  $P_i$ -ATP exchange be equal to or greater than the  $OP_i$ - $H_2O$  exchange. Data on incorporation into intramitochondrial reactants suggest that it is  $P_i$  and not ADP which initially loses its oxygen (19).

tion of a "high energy" compound or state prior to the formation of an intermediate from inorganic orthophosphate or adenosine diphosphate. In the following discussion the results are considered which support these statements.

Observations which suggest that the rapid exchange of  $P_i^{32}$  with ATP and of phosphate oxygen with water oxygen, as described herein, results from the dynamic reversal of a phosphate uptake reaction or reactions of oxidative phosphorylation follow. The rapid oxygen exchange has been found to occur only with preparations capable of rapid net oxidative phosphorylation, both exchange reactions are sensitive to known inhibitors of oxidative phosphorylation, particularly 2,4-dinitrophenol, the aging of mitochondria has a deleterious effect on the exchanges, analogous to its effect on net oxidative phosphorylation, the exchange reactions depend on the availability of high energy phosphate. No other known reactions offer as simple an explanation of the observations as does the reaction just described.

Evidence that at least two steps are involved in the formation of ATP from  $P_i$  and ADP comes from the much greater rate of the  $O_{P_i}$ - $H_2O$  exchange than the  $P_i$ -ATP exchange, clearly this would result if the steady state rate of a reaction responsible for the replacement of an oxygen of a phosphate compound was more rapid than that of the over-all reaction responsible for the incorporation of  $P_i$  into ATP. However, it must be recognized that the difference in exchange rates undoubtedly reflects in part the relative rapidity with which the  $P_i$  and the ATP of the medium exchange with that of the mitochondria. Compartmentalization of adenine nucleotides and of  $P_i$  in the mitochondria has been demonstrated by other workers, particularly Siekevitz and Potter (20). The observation, that the phosphate which is transferred to AMP or glucose in net oxidative phosphorylation contains less  $O^{18}$  than does the  $P_i$  of the medium, could logically result from compartmentalization of the  $P_i$  or the ADP in the mitochondria. Intramitochondrial  $P_i$  or ADP at the site of phosphate uptake could undergo several oxygen exchanges with water before appearing finally in the ATP, leaving the mitochondria.

The rapid interchange of  $P_i$  with the phosphates of ATP, as observed in these experiments, probably requires that the energy derived from a splitting of the ATP be retained in some manner for the reincorporation of the  $P_i$ . Retention of the energy by formation of an intermediate with ADP or AMP (e.g. an enzyme-ADP or enzyme-AMP) would explain the exchange data obtained with  $P^{32}$  but not with  $O^{18}$ . The  $O^{18}$  exchange requires that any intermediate containing the ADP, the AMP, or the phosphate moiety from the ATP must be reversibly cleaved by either  $H_2O$  or an oxygen compound which in turn derives its oxygen from  $H_2O$ . The

energy for reformation of the phosphorylated intermediate does not appear to be preserved by reduction of the electron carriers. Reaction conditions leading to nearly complete reduction of the electron carriers, and therefore to the lack of potential electron acceptors, did not decrease the amount of  $O^{15}$  or  $P^{32}$  exchange. These considerations support the suggestion that the energy is preserved as a high energy compound or state involving neither  $P_i$  nor the adenine nucleotides, and that the formation of such an energetic precursor to  $P_i$  uptake may be coupled to electron transport during oxygen uptake.

Recently, Cohn and Drysdale (4) have presented valuable evidence that in oxidative phosphorylation which accompanies  $\beta$ -hydroxybutyrate oxidation, phosphate undergoes additional oxygen exchange after the initial uptake reaction. They suggested the participation of an intermediate which supplies its oxygen to the phosphate of ATP, the oxygen of the intermediate eventually being replaced by the oxygen of water. However, consideration of more recent findings leads to the conclusion that the simplest explanation for the rapid exchange of phosphate oxygen with water oxygen is as given previously (1), namely the reversal of  $P_i$  uptake reaction or reactions of the over-all process of oxidative phosphorylation. The recent report of Siekevitz and Potter (21) on the concentrations of intramitochondrial nucleotides suggests that an alternative explanation of Cohn and Drysdale's findings should be considered. Calculations from the data of Cohn and Drysdale (4) show that the requisite moles of the postulated intermediate would be roughly 20 times the total number of moles of adenine nucleotides from the mitochondria used. Adenine nucleotides are a major constituent among the acid-extractable component from mitochondria (21). Thus, even if the intermediate were present in amounts equivalent to the adenine nucleotides, there would be insufficient to account for the oxygen supplied to the ATP. One alternative possibility is that components added to the reaction medium participate in the exchange. This is made plausible by the finding that the formation of acetyl coenzyme A is accompanied by transfer of an oxygen from the acetate carboxyl to the phosphate of the AMP formed from ATP (17). The  $\beta$ -hydroxybutyrate thiokinase reaction very likely proceeds with oxygen transfer analogous to the acetate thiokinase reaction. Thus oxygen from the AMP phosphate and from the  $\beta$ -hydroxybutyrate carboxyls intermix with other phosphate oxygens and appear in the phosphate of the ATP as a result of a dynamic state of the thiokinase reaction which acts together with reactions which form ADP and ATP from adenine monophosphate.<sup>7</sup> Exchange of phosphate oxygen with oxygens other than

<sup>7</sup> A dynamic state of the  $\beta$ -hydroxybutyrate thiokinase reaction would demand that intramitochondrial pyrophosphate exchange rapidly with the terminal phosphate.

water oxygen could thus be expected to occur in the early part of the incubation

The data presented herein are also pertinent to the use of the incorporation of  $P_i^{32}$  into ATP as a quantitative measure of net oxidative phosphorylation (22, 23). Although results which have been reported with the use of such techniques may be valid because of suppression of the exchange reaction under the conditions used, the quantitative measurement of net oxidative phosphorylation by  $P_i^{32}$  incorporation should be undertaken with caution. The marked inhibition of the exchange reaction by AMP and ADP may account for the lack of incorporation in many experiments made without added substrate. The presence of hexokinase or of ATPase together with adenylate kinase would favor formation of ADP and AMP from ATP. However, when substrate is present, attainment of relatively high ATP concentrations could quite conceivably lead to appreciable exchange of  $P_i$  with the phosphates of ATP and thus erroneously high "P/O" ratios, measurement of the amount of exchange without added substrate may thus not form a satisfactory control. From data reported in this paper, it is clear that, with appropriate conditions, P/O ratios approaching infinity could be obtained with the use of the extent of incorporation of  $P_i^{32}$  into adenosine triphosphate as a measure of oxidative phosphorylation.

#### SUMMARY

A rapid exchange of inorganic phosphate- $P^{32}$  with the phosphate groups of adenine nucleotides and an exchange of phosphate oxygens with those of water are catalyzed by liver mitochondria in the absence of added substrate and oxygen uptake. Some 15 to 20 phosphate oxygen atoms exchange for each exchange of inorganic phosphate with the adenine nucleotides of the medium. Both exchanges are inhibited by low concentrations of 2,4-dinitrophenol. The  $P^{32}$  exchange does not result from the action of ATPase and does not occur with uridine triphosphate or inosine triphosphate in place of adenosine triphosphate.

The presence of anaerobic conditions or of  $10^{-4}$  M cyanide, with or without added succinate, has little effect on the extent of both exchange reactions. The incubation of mitochondria at  $37^\circ$  for short periods with 2,4-dinitrophenol or for longer periods without 2,4-dinitrophenol results in a loss of ability to catalyze the oxygen exchange, the addition of ATP partially restores the exchange.

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phate of ATP. Support for this possibility comes from the recent finding (19) that pyrophosphate of the reaction medium may exchange rapidly with the terminal phosphate of ATP in the presence of mitochondria.

Phosphate which is taken up in oxidative phosphorylation and which is transferred to glucose or to adenylic acid shows a more extensive oxygen exchange than does the inorganic phosphate of the medium

Phosphate oxygen exchange occurs readily in the intact rat, but no exchange between the oxygens of water and those of phosphate was detected with *Leuconostoc mesenteroides* P-60

The data suggest that both exchanges result from the dynamic reversal of the phosphate uptake and transfer reactions associated with the overall process of oxidative phosphorylation, that at least two steps are involved in the formation of ATP, the first of which is responsible for the oxygen exchange reaction, and that energy derived from oxidation of electron carriers of ATP cleavage can be preserved in some manner other than as a compound which is derived from adenine nucleotides or inorganic phosphate

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# METABOLIC INHOMOGENEITY IN RIBONUCLEIC ACID PHOSPHORUS OF PARTICULATE FRACTIONS OF LIVER\*

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There is abundant evidence of intracellular inhomogeneity in the turnover of the phosphorus component of liver ribonucleic acid (RNA). Several investigators (1-4) have shown that the P of nuclear RNA has a much higher turnover rate than the P of cytoplasmic RNA. Smellie *et al* (5) have carried the analysis further and shown that the turnover rate of the P of the cytoplasmic soluble RNA has a higher turnover rate than does the P of RNA of mitochondria or microsomes. However, within each particulate fraction, the turnover rates of the P of the individual mononucleotides showed only very minor differences. Smellie *et al* isolated the four mononucleotides after hydrolysis of the RNA by the Schmidt-Thannhauser (6) procedure and found them to have practically equal specific activities. Moldave and Heidelberger (4) used ribonuclease to separate the RNA isolated from liver particulate fractions into a dialyzable fraction and a non-dialyzable core resistant to the action of this enzyme. In livers isolated from animals that had been injected with  $P^{32}$ -phosphate, orotic acid-6-C-14, or glycine-2-C-14, the specific activity of the dialyzable portion was 2- to 3-fold that of the resistant core.

Experiments have been reported from this laboratory (7) in which the total RNA of livers removed from animals previously injected with  $P^{32}$ -phosphate was subjected to fractional degradation by treatment with successive portions of a 5 per cent solution of trichloroacetic acid (TCA) at room temperature. The mono- and oligonucleotide fractions separated from the water-soluble products by ion exchange chromatography were found to show a wide range of relative specific activities. The variations in specific activity were greater than those between nuclear and cytoplasmic RNA reported by Smellie *et al* (5). The possibility could not be ruled out that the fragments of highest specific activity were derived from nuclear RNA and those of lowest specific activity from some cytoplasmic portion. Experiments were therefore carried out in which the particulate

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fractions of liver were separated by differential centrifugation, the RNA in each particulate fraction subjected to the fractional degradation by TCA and the products separated by ion exchange chromatography. Quantitative differences in the turnover rate were found, although the extremes were somewhat less than those in the previous experiments. Within each particulate fraction, at least a 10-fold range was found between the highest and the lowest specific activities.

#### EXPERIMENTAL

The experiments were carried out on rats and rabbits. 2 hours after the injection of the tracer phosphate, the liver was excised under anesthesia and immediately plunged into a large volume of ice-cold 0.25 M solution of sucrose. Each rabbit liver was worked up separately, the livers of four rats were pooled. The livers were cut into small pieces and the bulk of the erythrocytes was washed out by repeated shaking with the ice-cold sucrose solution. The rabbit livers were squeezed through stainless steel screens and then homogenized for 2 minutes in a Potter-Elvehjem homogenizer with a Teflon pestle. The rat livers were homogenized without the preliminary passage through the screens. These operations and the subsequent centrifugations were carried out in a cold room maintained at  $1-2^{\circ}$  or in a refrigerated centrifuge operating at this temperature. The homogenate was centrifuged at  $150 \times g$  for 10 minutes, the residue again homogenized for an additional 2 minutes, and again centrifuged at  $150 \times g$ . This residue was shaken with a small portion of the sucrose solution and again centrifuged at  $150 \times g$ . This procedure left behind a considerable amount of cell debris and erythrocytes and also involved the loss of some liver cell nuclei. The supernatant fluids from the centrifugations at  $150 \times g$  were layered over a 0.34 M sucrose solution, as described by Hogeboom *et al.* (8), and centrifuged at  $900 \times g$  for 10 minutes to isolate the nuclear fraction. Sufficient 1 M  $\text{CaCl}_2$  solution was added to the combined supernatant fluids to give a concentration of 0.0018 M (8), and this was then centrifuged at  $8500 \times g$  for 20 minutes to give the mitochondrial fraction. The microsomes were separated by centrifugation for 30 minutes at  $25,000 \times g$  (5).

Each sediment was suspended in a small volume of 0.25 M sucrose solution, and to these and to the cytoplasmic soluble fraction was added enough 50 per cent solution of TCA to give a final concentration of 10 per cent. Separation of the acid-soluble P and fractional degradation of the RNA with TCA were carried out as in the previous experiments on whole liver (7). The removal of TCA, adsorption of the nucleotide materials on columns of Dowex 1 anion exchange resin, and elution of the nucleotide fractions were made as before, with one modification. The ion exchange

umns were jacketed, and water at 1-3° was circulated through the jackets during the entire elution period. This was done to minimize any hydrolysis of oligonucleotide fractions during the elution in the hope that the oligonucleotide fractions isolated would be more clearly defined entities than was the case in the previous experiments. The eluents used were those of Cohn (9) for the mononucleotides: 0.001 N HCl for cytidylic acid, 0.002 N HCl for adenylic acid, 0.003 N HCl for uridylic acid, and 0.005 N HCl for guanylic acid. These were followed by 0.01 N HCl, 0.01 N HCl plus 0.05 M NaCl, 0.01 N HCl plus 0.1 M NaCl, and the columns were finally stripped with 2 N HCl. The separate fractions obtained were treated as previously for the isolation and determination of P and for the preparation and counting of the samples. In most cases the amount of P

TABLE I

*Degradation of Particulate Fractions of Rabbit Liver RNA by 5 Per Cent Trichloroacetic Acid Solution*

Each extract was made up to 100 ml after removal of TCA and the optical density was determined at 260 and 280 m $\mu$ .

Extract No	Duration of extraction	Nuclei		Mitochondria		Microsomes		Cytoplasmic soluble	
		260 m $\mu$	280 m $\mu$	260 m $\mu$	280 m $\mu$	260 m $\mu$	280 m $\mu$	260 m $\mu$	280 m $\mu$
	<i>hrs</i>								
1	2	0.095	0.062	0.250	0.145	0.40	0.250	1.45	0.80
2	24	0.755	0.455	4.10	2.30	5.50	3.05	2.25	1.40
3	24	0.60	0.40	2.55	1.45	4.10	2.35	2.0	1.10
4	48	0.205	0.145	0.540	0.300	0.263	0.143	0.920	0.550

present was sufficient for determination by the method of Fiske and Subbarow (10), but in some cases, particularly those representing fractions of nuclear RNA, the total quantity of P present was so small that recourse to the method of Berenblum and Chain (11) was necessary.

#### RESULTS AND DISCUSSION

The fractional degradation of the RNA of each of the particulate fractions yielded results comparable to those obtained on whole liver. In each case the first extraction, for 2 hours, yielded a relatively small amount of material, and the largest amount was found in the second extraction, for 24 hours. Only in the case of the cytoplasmic soluble RNA did the first extraction yield enough nucleotide material for further fractionation by ion exchange procedure. Table I presents the data on the amounts of nucleotide material obtained from the liver of a rabbit.

Well defined peaks in the elution pattern corresponding to cytidylic

acid in ratio of optical densities at 280 and 260  $m\mu$  were obtained in every extract subjected to ion exchange chromatography. A few peaks corresponding to each of the other mononucleotides were obtained occasionally. Also, as in the earlier work on whole liver, the nucleotide material eluted with any particular eluent did not necessarily have a constant composition with respect to ratio of optical densities at 280 and 260  $m\mu$ . In the last portion of the RNA rendered soluble by the treatment with TCA, the amount of P obtained by wet ashing of the cytidylic acid fraction was many times that calculated from the optical density. This excess P probably represents the substances which Smellie *et al* (5) found in separation of the products of their Schmidt-Thannhauser digests by paper electrophoresis. Two spots on the radioautographs of this material showed no absorption in the ultraviolet region. No such excess of P was found in any fractions of the nuclear RNA.

The radioactivity data from a single experiment on a rabbit are shown in Table II, and those from a pool of four rat livers are shown in Table III. In the latter experiment, the cytoplasmic soluble material was not processed. In this experiment, as in the others on rat livers, the total nucleotide material present in any one TCA extract of nuclei was too small for satisfactory resolution by the ion exchange resin. The total extract was therefore wet ashed for conversion to orthophosphate and preparation of the samples for counting.

It is evident that there is a fairly wide range in the specific activities of the portions of nucleotide material isolated from any one particulate fraction, and within the degradation products rendered soluble by one treatment with TCA. Within any one particulate fraction, except for the nuclear RNA from rat liver, the range of specific activities is greater than that found by Moldave and Heidelberger (4) between that portion hydrolyzed by ribonuclease and the enzyme-resistant core. The present experiments therefore emphasize their conclusion of a metabolic inhomogeneity within the RNA of any particulate fraction of liver. These data also show that the very high degree of metabolic inhomogeneity that was previously reported for the RNA of whole liver (7) cannot be explained on the basis of high specific activities of fragments derived from nuclear RNA and low specific activities of fragments of cytoplasmic origin.

The present findings call for a reinterpretation of the results of experiments in which the specific activity of the entire nuclear RNA-P was compared with that of entire cytoplasmic RNA-P or with that of the total RNA-P of any cytoplasmic particulate fraction. On the basis of these experiments, Marshak and Calvet (2) and Jeener and Szafarz (3) concluded that nuclear RNA was the precursor of cytoplasmic RNA. The data of Smellie *et al* (5) are considered by them not to preclude such an interpretation.

tion but to indicate that, if such a transfer of RNA from nucleus to cytoplasm does take place, it is not by simple diffusion

TABLE II  
*Relative Specific Activities of P from Fragments of RNA of  
Particulate Fractions of Rabbit Liver*

Liver of rabbit excised 2 hours after injection of  $P^{32}$ -phosphate The values are in terms of counts per minute per mg of P, calculated to a basis of  $10^6$  c p m injected per kilo of body weight

Extract No	Nuclei	Mitochondria	Microsomes	Cytoplasmic soluble
1	3595 (t)	780 (t)	108 (t)	31 (a) (Cyt ) 11 (d) 24 (e) 269 (f) 39 (g) 40 (h)
2	96 (a) (Cyt ) 140 (e) 149 (f) 84 (g) 116 (h)	144 (a) (Cyt ) 29 (d) 19 (e) 40 (f) 34 (g) 10 (h)	44 (a) (Cyt ) 187 (d) 16 (e) 30 (f) 32 (g) 10 (h)	25 (a) (Cyt ) 28 (b) (Ad ) 18 (c) 23 (d) 27 (e) 45 (f) 32 (g) 23 (h)
3	52 (t)	73 (a)* (Cyt ) 53 (c) (Urid ) 22 (e) 64 (f) 31 (h)	10 (a) (Cyt ) 46 (b) 27 (c) 37 (d) (Guan ) 172 (f) 16 (h)	21 (a)* (Cyt ) 23 (c) 24 (e) 10 (f) 45 (h)
4	47 (t)	39 (t)*	41 (t)*	39 (t)*

The letters in parentheses signify the eluent with which this fraction was obtained (a) 0.001 N HCl, (b) 0.002 N HCl, (c) 0.003 N HCl, (d) 0.005 N HCl, (e) 0.01 N HCl, (f) 0.01 N HCl plus 0.05 M NaCl, (g) 0.01 N HCl plus 0.1 M NaCl, (h) 2 N HCl, (t) entire extract, not fractionated Cyt, cytidylic acid, Ad, adenylic acid, Guan, guanylic acid, Urid, uridylic acid

\* Signifies presence of non-nucleotide P

Such an interpretation would be reasonable if it could be shown that the turnover rate of all parts of the molecule of RNA within the nucleus were higher than the observed turnover rate of all parts of the RNA within any cytoplasmic particulate fraction, or within the cytoplasmic soluble RNA. The requisite condition for such a showing would be that, during the rising phase of specific activity of nuclear RNA, no portion of any cytoplasmic

RNA should be found with a higher specific activity than any fragment of nuclear RNA. Smellie *et al* (5) found that the specific activity of nuclear RNA reaches a maximum 30 hours after the injection of the tracer phosphate. In the present experiments, the livers were excised 2 hr after the injection of the tracer. Since some fragments of mitochondrial, microsomal, and cytoplasmic soluble RNA in the present experiments were found to have specific activities appreciably higher than some fragments

TABLE III

*Relative Specific Activities of P from Fragments of RNA of Particulate Fractions of Rat Liver*

Pooled livers of four rats, excised 2 hours after injection of  $P^{32}$ -phosphate. P values are in terms of counts per minute per mg of P, calculated to a basis of 1 c p m injected per kilo of body weight

Extract No	Nuclei	Mitochondria	Microsomes
1	332 (t)	420 (t)	308 (t)
2	388 (t)	98 (e) 82 (f) 25 (g) 20 (h)	68 (a) (Cyt) 33 (e) 41 (f) 23 (f) 48 (f) 157 (g) 28 (h)
3	537 (t)	31 (e) 66 (f) 32 (f) 68 (g) 32 (h)	42 (e) 95 (f) 115 (g) 31 (h)
4	542 (t)	1175 (t)	755 (t)

The letters in parentheses and the abbreviation have the same significance as in Table II. Cyt = cytidylic acid

of nuclear RNA, it is evident that nuclear RNA cannot be the direct precursor of the phosphate groups of cytoplasmic RNA. Whether the same situation holds for *de novo* synthesis of the purine and pyrimidine bases remains to be established by appropriate experiments with  $C^{14}$ -labeled precursors. The data of Moldave and Heidelberger (4) suggest that such is also the case. Certainly, with respect to turnover of phosphate groups, it is clear that this process takes place independently within the nucleus and within each of the particulate fractions in the cytoplasm. There are great variations in the turnover rate of different portions of the RNA macromolecule, with the average rate in the nucleus being much higher than the average rate in mitochondria, microsomes, or cytoplasmic soluble

RNA The failure of Smellie *et al* (5) to obtain a simple precursor-product relation in the time-course of specific activities of nuclear and cytoplasmic RNA can be accounted for by the present findings The combination of the rapid fall in specific activity of the intracellular acid-soluble P with time, the high turnover rate of a portion of the nuclear RNA, and the great differentials in turnover rate within nuclear and cytoplasmic RNA all combine to make such a simple relation improbable

#### SUMMARY

1 The ribonucleic acid (RNA) of the particulate fractions of liver of rats and rabbits previously injected with tracer phosphate has been subjected to fractional degradation with dilute trichloroacetic acid solution at room temperature

2 The water-soluble fragments resulting from such treatment of any one particulate fraction have been found to show wide ranges in turnover rates of their phosphate groups

3 Some fragments of RNA from mitochondria, microsomes, and cytoplasmic soluble RNA were found to show higher turnover rates than some fragments of nuclear RNA

4 The data obtained indicate that turnover of RNA phosphorus takes place independently in each of the particulate fractions, and that nuclear RNA is not the direct precursor of the phosphate groups of the RNA of the cytoplasmic particulate fractions

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# EFFECTS OF BUTYL-4-HYDROXY-3,5-DIODOBENZOATE ON THE METABOLISM OF $I^{131}$ -THYROXINE AND TRIIODOTHYRONINE\*

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Comparatively little is known about the interference in actions and metabolism of hormones by their appropriate analogues, though thyroxine has been studied in this regard more than have other hormones. One analogue which has been demonstrated by MacLagan *et al* (1) to prevent the calorigenic effect of thyroxine is butyl-4-hydroxy-3,5-diiodobenzoate (BHDB). The mechanism of antagonism has not been elucidated, but the effects of this antagonist on thyroxine distribution, degradation, and excretion seemed worthy of study. The effects of BHDB on triiodothyronine metabolism also would be important to know, since, even though the actions of triiodothyronine appear to be very similar to those of thyroxine in kind (2), there are significant quantitative differences in their activities and contrasts in their metabolism (3-6). In general, triiodothyronine is more rapidly accumulated by tissues *in vivo*, it exerts a more potent and rapid calorigenic effect, it is degraded more rapidly, and its effect on oxygen consumption is more transient.

Certain observations have been reported previously on the effect of BHDB on degradation of the  $I^{131}$ -labeled hormones. In rats, Wilkinson *et al* found that the degradation of both thyroxine and triiodothyronine, as measured by the rate of urinary excretion of radioactivity, was inhibited by BHDB (7, 8). These findings were contrary to those reported by Roche, Deltour, and Michel for  $I^{131}$ -thyroxine and BHDB (9), but, as later demonstrated by Wilkinson and Feetham (10), the findings of Roche's group could be explained by the fact that administration of BHDB was not continued throughout the experiment.

The studies reported herein confirm the observations of Wilkinson *et al* (7, 8) on the excretion pattern of radioactivity following the administration of  $I^{131}$ -thyroxine and  $I^{131}$ -triiodothyronine to rats and also demon-

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strate that BHDB produces alterations in the distribution of these thyroid hormones in blood and in certain tissues

### EXPERIMENTAL

In all experiments, female Sprague-Dawley rats, bred locally, were used. They weighed from 190 to 220 gm and were maintained on a standard Purina fox chow diet prior to each experiment

#### *Precipitability of $I^{131}$ -Thyroxine and $I^{131}$ -Triiodothyronine*

Since assumptions are made in this study that trichloroacetic acid soluble radioactivity represents almost entirely the degradation products (p-iodotyrosines, iodide) of thyroxine and triiodothyronine, it was necessary to demonstrate the degree to which the labeled hormones are precipitated by trichloroacetic acid (TCA) in the presence of animal proteins. In the presence of the latter the precipitation of the hormones is more complete, particularly when their concentration is low. However, even in the absence of protein TCA precipitates a significant portion of these hormones.

Liver and whole blood were removed from fasted normal animals under ether anesthesia. A 10 per cent homogenate of liver in water was prepared and separated into twenty 2.5 ml aliquots. The heparinized whole blood was obtained in 3 ml amounts from twelve separate animals.

0.1 ml amounts of  $I^{131}$ -thyroxine,<sup>1</sup> each containing 0.004  $\gamma$  (0.2  $\mu$ g) were added to each of ten liver homogenate preparations, followed immediately by 2.5 ml of 10 per cent TCA. The same amount of labeled thyroxine was added to each of six blood samples, followed immediately by 3 ml of 20 per cent TCA.

The remainder of the liver and blood samples were treated similarly.  $I^{131}$ -labeled triiodothyronine<sup>1</sup> (0.0035  $\gamma$  in 0.2  $\mu$ c) being used instead of thyroxine.

All specimens were then stirred carefully and centrifuged, and the precipitates were washed twice with 10 per cent TCA. The precipitates were dissolved in 30 per cent KOH, the resulting TCA-soluble and TCA-insoluble fractions were then diluted to 30 ml, and the radioactivity assayed in a well type  $\gamma$  counter.<sup>2</sup>

#### *Effect of BHDB on Tissue Distribution of $I^{131}$ -Thyroxine and $I^{131}$ -Triiodothyronine*

This portion of the study was done in order to determine whether BHDB would tend to displace hormones from tissue sites and thus produce alterations.

<sup>1</sup> L-Thyroxine and L-3,5,3'-triiodothyronine, labeled with  $I^{131}$  in the position adjacent to the hydroxyl group (11), were obtained from the Abbott Laboratories, Oak Park, Illinois, Tennessee.

<sup>2</sup> Obtained from the Welch-Allyn Corporation, Skaneateles Falls, New York.

tions in the concentration of these substances in representative tissues and blood

Ten rats received BHDB suspended in a 0.2 N sodium carbonate solution, adjusted to pH 7.8 as described by Wilkinson *et al* (7), 25 mg were administered in 1 ml portions subcutaneously on the day preceding, and again 1 hour before, the start of the experiment<sup>3</sup>. These animals and twelve other rats, used as controls, were fasted for 14 hours prior to the experiment. Five BHDB-treated and six control animals received 0.2  $\gamma$  (5  $\mu\text{c}$ ) of  $\text{I}^{131}$ -thyroxine injected into a caudal vein. The remaining animals received 0.16  $\gamma$  (5  $\mu\text{c}$ ) of  $\text{I}^{131}$ -triiodothyronine in a similar fashion. Animals were sacrificed by exsanguination either 1 or 24 hours following administration of the labeled hormones. Whole blood was drawn from the abdominal aorta into heparinized syringes, and specimens of liver, kidney, and gastrocnemius muscle were also removed. 3 ml of blood were immediately mixed with an equal volume of 20 per cent TCA, the tissues were weighed and homogenized in 10 per cent TCA. After centrifugation, the precipitates were washed twice with 10 per cent TCA and then dissolved in 30 per cent KOH. The TCA-soluble and TCA-insoluble fractions were then radioassayed as described above.

*Effect of BHDB on Protein Binding of  $\text{I}^{131}$ -Thyroxine As Revealed by Paper Electrophoresis and Autoradiographic Studies*

Certain human serum proteins, particularly albumin and some  $\alpha$ -globulins, are able to bind thyroxine, as demonstrated by paper electrophoresis (12, 13). We were interested in ascertaining whether BHDB could compete successfully with thyroxine for similar binding sites in the rat. Therefore, we designed an experiment whereby the effect of BHDB on the electrophoretic migration of  $\text{I}^{131}$ -thyroxine radioactivity with serum proteins could be observed.

Five 200 gm rats were given 25 mg of BHDB subcutaneously. 4 hours later these and five similar control animals were given 4  $\gamma$  (100  $\mu\text{c}$ ) of labeled thyroxine intravenously. This relatively large amount of thyroxine was used in order to obtain sufficient radioactivity in serum for autoradiographic purposes. After 15 minutes, aortic blood was obtained from each animal and allowed to clot in a test tube, the blood was then centrifuged and the serum was removed. 0.2 mg of BHDB was added to a 1 ml aliquot of serum from the control rats. The remainder of the control sera was used unchanged. In summary, there were five specimens for each of the following three groups: (a)  $\text{I}^{131}$ -thyroxine injected intravenously into animals pretreated with BHDB, (b)  $\text{I}^{131}$ -thyroxine injected intra-

<sup>3</sup> The butyl-4-hydroxy-3,5-diiodobenzoate was generously supplied by Professor N F MacLagan, Department of Chemical Pathology, Westminster Medical School, London, South West 1, England.

radioactivity to  $\alpha$ -globulin was not appreciably altered. When added directly to normal serum obtained from animals given only  $I^{131}$ -thyroxine, BHDB displaced radioactivity from the albumin zone.

TABLE II  
Radioactivity in Rat Tissues Following Administration of  $I^{131}$  Labeled Thyroxine to BHDB-Treated and Control Rats

Tissue	Time after injection of $I^{131}$ -thyroxine	No. and group of rats	TCA-soluble fraction			TCA precipitated fraction		
			(A)*	(B)†	(C)‡	(A)*	(B)†	(C)‡
Blood	hrs	6 control 5 BHDB	495	0 10	0 12 $\pm$ 0 02	12,952	2 71	3 25 $\pm$ 0 07
			248	0 05	0 06 $\pm$ 0 01	6,552	1 35	1 70 $\pm$ 0 01
P§					<0 001			<0 001
	24	4 control 5 BHDB	173	0 04	0 08 $\pm$ 0 02	2,371	0 51	1 04 $\pm$ 0 01
			70	0 02	0 03 $\pm$ 0 01	715	0 15	0 31 $\pm$ 0 01
P					<0 001			<0 001
Liver	1	6 control 5 BHDB	296	0 28	0 34 $\pm$ 0 01	7,752	7 49	8 99 $\pm$ 0 07
			330	0 28	0 35 $\pm$ 0 05	10,294	8 91	11 11 $\pm$ 1
P					>0 5			<0 01
	24	5 control 5 BHDB	146	0 05	0 09 $\pm$ 0 02	3,245	1 09	2 22 $\pm$ 0 01
			104	0 03	0 06 $\pm$ 0 01	3,316	1 03	2 11 $\pm$ 0
P					<0 05			>0 5
Kidney	1	6 control 5 BHDB	195	0 23	0 27 $\pm$ 0 04	1,975	2 29	2 73 $\pm$ 0 07
			173	0 20	0 25 $\pm$ 0 04	2,302	2 54	3 18 $\pm$ 0 07
P					>0 5			<0 02
			Total radioactivity					
			(A)*		(B)†	(C)‡		
Muscle	1	6 control 5 BHDB	408		0 29	0 30 $\pm$ 0 01		
			335		0 20	0 29 $\pm$ 0 01		

\* (A), counts per minute in fraction (mean value)

† (B), per cent of administered dose per gm. of tissue (mean value)

‡ (C), (B)  $\times$  body weight of rat per 100 (mean value  $\pm$  standard deviation)

§  $P$ , the probability that paired data in (C) (BHDB versus control) show differences due to chance alone

The excretion of radioactivity into the urine and intestine, following labeled hormone administration, is shown in Fig 2 The rate of urinary

TABLE III  
*Radioactivity in Rat Tissues Following Administration of  $I^{131}$ -Labeled  
Thiodothyronine to BHDB-Treated and Control Rats*

Tissue	Time after injection of $I^{131}$ -thiodothyronine	No and group of rats	TCA soluble fraction			TCA precipitated fraction		
			(A)*	(B)†	(C)‡	(A)*	(B)†	(C)‡
Blood	hrs 1	6 control 5 BHDB	360	0 08	0 12 $\pm$ 0 02	2024	0 42	0 64 $\pm$ 0 05
			116	0 03	0 04 $\pm$ 0 00	1723	0 36	0 55 $\pm$ 0 02
P§					<0 001		<0 02	
	24	5 control 5 BHDB	976	0 12	0 27 $\pm$ 0 08	1221	0 16	0 34 $\pm$ 0 16
			182	0 03	0 06 $\pm$ 0 03	539	0 08	0 18 $\pm$ 0 05
P					<0 001		<0 01	
Liver	1	6 control 5 BHDB	471	0 48	0 74 $\pm$ 0 07	3204	3 36	5 06 $\pm$ 0 37
			564	0 56	0 85 $\pm$ 0 17	3710	3 61	5 45 $\pm$ 0 40
P					<0 2		<0 2	
	24	5 control 5 BHDB	1223	0 12	0 21 $\pm$ 0 01	3817	0 31	0 55 $\pm$ 0 17
			412	0 03	0 07 $\pm$ 0 02	6487	0 52	1 12 $\pm$ 0 16
P					<0 001		<0 001	
Kidney	1	6 control 5 BHDB	798	0 85	1 28 $\pm$ 0 23	3821	4 12	6 20 $\pm$ 0 61
			656	0 68	1 02 $\pm$ 0 15	4001	4 09	6 18 $\pm$ 0 49
P					<0 1		>0 5	
			Total radioactivity					
			(A)*		(B)†	(C)‡		
Muscle	1	6 control 5 BHDB	1026		0 44	0 66 $\pm$ 0 09		
			916		0 42	0 63 $\pm$ 0 10		

\* , † , ‡ , § See corresponding footnotes in Table II

excretion of radioactivity was greater following labeled thiodothyronine than following labeled thyroxine administration BHDB depressed the urinary excretion of radioactivity after the administration of either hor-

mone, but it caused a proportionately greater increase in the amount of intestinal and fecal radioactivity. BHDB did not alter the fraction of fecal radioactivity which was TCA-precipitable (approximately 90 %).

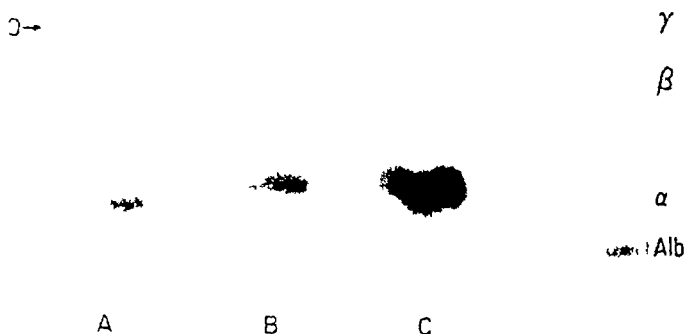


FIG. 1. Filter paper electrophoresis of rat sera. The strip on the right shows a typical protein pattern of serum stained with amidoschwarz 10B. The other three strips are autoradiographs. Strip A is representative of the pattern obtained with serum from a normal animal given labeled thyroxine. Strip C shows the pattern obtained when BHDB is added to this same serum. Strip B is representative of the pattern obtained with serum from a BHDB-treated animal given labeled thyroxine. 0 indicates the point of application of the serum for all four strips. Note in Strip B and Strip C the absence of radioactivity in the albumin zone.

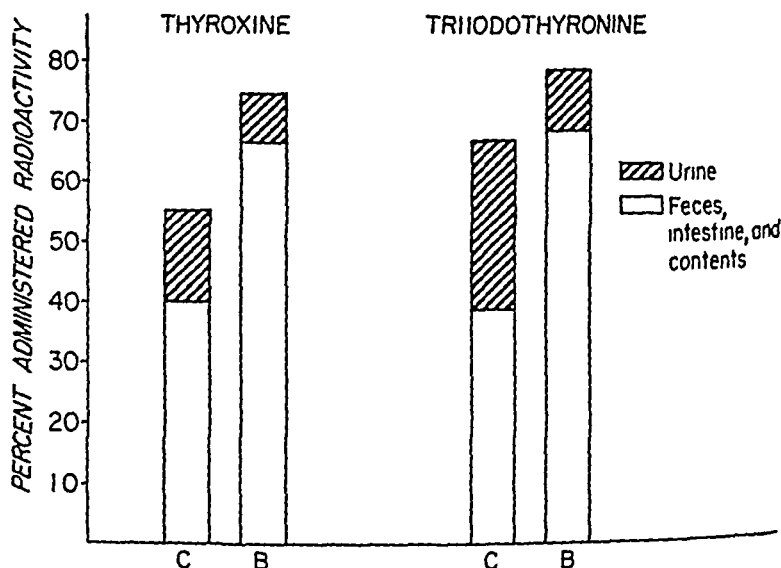


FIG. 2. Note in the BHDB-treated animals the markedly increased fecal excretion and the less marked drop in urinary excretion of radioactivity over a 24 hour period following the administration of  $I^{131}$ -labeled thyroxine and triiodothyronine. In the controls that received  $I^{131}$ -triiodothyronine is observed a larger excretion of radioactivity in the urine than in the controls that received  $I^{131}$  thyroxine. Strip C, control (five animals), Strip B, BHDB-treated (five animals).

cent), but the absolute amount of fecal, TCA-precipitable radioactivity was increased to the same proportion as was the total radioactivity. The amount of TCA-precipitable radioactivity in the urine remained negligible, and the urinary volumes and fecal weights were not changed significantly by the presence of BHDB during the 24 hours of this study.

#### DISCUSSION

With the comparatively large doses used, BHDB exerted a potent inhibitory effect on the rate of degradation of both labeled hormones. The degree of depression in urinary excretion of radioactivity was quite similar to that noted by Wilkinson *et al* (7, 8). Since the labeled hormones are almost entirely precipitable by TCA (Table I), it may be assumed that much of the TCA-soluble radioactivity recovered from animals injected with these hormones represented products of degradation. It was of considerable importance, therefore, that the percentage of TCA-soluble radioactivity in the blood and, at 24 hours, in the liver was lower in the BHDB-treated animals than in the control group, thus indicating that less conversion of hormone to a TCA-soluble product occurred.

The ability of BHDB to produce a lowering of TCA-precipitable radioactivity in the blood was especially marked following the administration of labeled thyroxine. This was totally unexpected, because one might predict that blood radioactivity would be definitely higher in the BHDB group if impaired degradation were the only factor involved. The autoradiographs of the electrophoretic patterns (Fig. 1) show that the low levels of blood radioactivity might be due to absence of binding of  $I^{131}$ -thyroxine to serum albumin in the presence of BHDB, hence only the  $\alpha$ -globulin was available for such binding and the amount of hormone bound by this globulin appears unaltered. In normal human serum  $\alpha$ -globulin is the main binding site for thyroxine (12, 13), but some binding to albumin is found even at the lowest concentrations studied (13). Moreover, albumin is the "main secondary carrier." When  $I^{131}$ -thyroxine is incubated in normal or myxedema serum in concentrations of 50  $\gamma$  per cent most of the thyroxine is found in the albumin fraction (16).

Even though triiodothyronine is normally bound only slightly to  $\alpha$ -globulin and not at all to albumin in human serum (13), BHDB also depressed the blood TCA-precipitable levels of this hormone, but the effect was much less marked.

The increased amount of TCA-precipitable radioactivity in the intestinal tract and feces was not necessarily a primary effect of BHDB. It may, rather, have been a homeostatic reaction, possibly involving an increased rate of biliary excretion of TCA-precipitable radioactivity, ridding the organism of hormone which would otherwise accumulate, due to the di-

minished rate of degradation. It should be noticed, however, that the total excreted radioactivity, plus that remaining in the intestine at the end of 24 hours following the administration of either labeled hormone was considerably greater in the BHDB group than in the controls in this study (Fig. 2). This may well have been a result of the impaired binding of hormone to serum albumin. Two mechanisms, then, seem to have been operating to increase the excretion of radioactivity in the feces when BHDB was administered.

The only instance in which BHDB produced an effect on triiodothyronine metabolism qualitatively different from that on thyroxine metabolism is represented by the precipitable radioactivity in the liver (Tables II and III). In the presence of BHDB the thyroxine radioactivity was elevated at 1 hour, but unaffected at 24 hours. The opposite was true of triiodothyronine radioactivity. The early elevation of TCA-precipitable liver (and kidney) radioactivity following thyroxine administration is best explained by the deficiency in binding to serum albumin, with the result that radioactivity was more rapidly transferred to the tissues. However, we have no explanation for the even more marked increase in liver TCA-precipitable radioactivity 24 hours after labeled triiodothyronine administration.

Since BHDB was administered in doses which were over 20,000 times greater than the doses of labeled thyroxine, little can be said as to how effective BHDB is in competing with thyroxine and triiodothyronine for participation in enzymatic reactions. It seems significant that, in spite of the great molar excess of BHDB, and in spite of its marked inhibitory effect on the binding of  $I^{131}$ -thyroxine to serum albumin, we could not demonstrate that BHDB inhibited tissue binding of either hormone. Its apparent inhibition of hormone degradation may, therefore, involve more subtle mechanisms.

Although they could produce inhibition of the calorigenic effect of thyroxine by BHDB, MacLagan and coworkers were able to observe no such effect on the response to triiodothyronine; in fact, this response was slightly enhanced by BHDB (1). Thus, it is apparent that the processes of calorigenic action and degradation of triiodothyronine may involve separate enzymatic pathways, only one of which is affected by BHDB. For thyroxine, both of these processes are apparently blocked by the analog; thus, it is still possible that inhibition of the conversion of this hormone to triiodothyronine may be the mechanism responsible for inhibition of its calorigenic effect, if not of its degradation.

#### SUMMARY

The effects of butyl-4-hydroxy-3,5-diiodobenzoate (BHDB) on the tissue distribution and excretion patterns of radioactivity following the administration of

tration of thyroxine or triiodothyronine labeled with  $I^{131}$  have been studied in rats

Under the influence of BHDB, degradation of both labeled hormones was apparently slowed, as represented by a lower rate of excretion of TCA-soluble radioactivity in the urine, and by the presence of a lower percentage of TCA-soluble radioactivity in the blood and later in the liver. The amount of radioactivity found in the feces and intestines was greater in the BHDB-treated group. This more than compensated for the decreased urinary radioactivity over a 24 hour period and therefore explains both lower urine and blood activity.

BHDB also produced a marked depression in the TCA-precipitable radioactivity in the blood following  $I^{131}$ -thyroxine administration. This alteration was probably responsible for the higher concentration of liver and kidney TCA-precipitable radioactivity following labeled thyroxine administration to BHDB-treated animals. This could be attributed to the decreased binding of thyroxine to albumin, without significant alteration in binding to other proteins, as demonstrated by paper electrophoretic studies and autoradiographs.

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# SEPARATION OF $\Delta^4$ -5 $\alpha$ - AND $\Delta^4$ -5 $\beta$ -HYDROGENASES FROM RAT LIVER HOMOGENATES\*

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Previous studies with rat liver tissue and tissue preparations have demonstrated the presence of enzyme systems capable of reducing the  $\Delta^4$  grouping of various steroids to the 5 $\alpha$  or 5 $\beta$  stereoisomeric forms. The 5 $\alpha$  (allopregnane)-reduced products were identified after incubation of deoxycorticosterone with rat liver slices (1), perfusion of cortisone (2, 3) and cortisol (4) through rat livers, incubation of progesterone with a rat liver homogenate (5), and the incubation of 11-deoxycortisol with a supernatant fluid derived from a rat liver homogenate (6). That rat liver contains enzyme systems which reduce the  $\Delta^4$  group to the 5 $\beta$  form is evident from the incubation of cortisone with a supernatant fluid derived from a rat liver homogenate centrifuged at  $78,000 \times g$  (7). This communication reports the separation of the two reducing enzyme systems (5 $\alpha$  and 5 $\beta$ ) from a rat liver homogenate.

## *Materials and Methods*

The collection of tissue and the homogenization, extraction, isolation, and purification procedures employed in this study have been previously described (6).

Diphosphopyridine nucleotide (DPN) "90" was obtained from the Sigma Chemical Company, St. Louis. The potassium hexose diphosphate was the gift of Dr. E. Keller, and the steroid compounds were generously donated by Dr. K. Pfister and Dr. M. Tishler of Merck and Company, Inc.

## EXPERIMENTAL

### *Preliminary Experiment A*

In a preliminary experiment, Experiment A, rat liver tissue was homogenized in a Krebs-Ringer-phosphate buffer at pH 7.35 and separated into particulate and soluble fractions by differential centrifugation. The

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homogenate fractions were incubated with 11-deoxycortisol, the extracts of the incubation mixtures were chromatographed by means of paper chromatography, and an attempt was made to identify the ring A-reduced products. The results indicated a sharp difference in the steric course of ring A reduction between the soluble homogenate fraction and the particulate fractions (Table I). From the incubation of 11-deoxycortisol with the soluble fraction, the 5 $\beta$  derivative, 3 $\beta$ ,17 $\alpha$ ,21-trihydroxypregnan-20-one, was isolated and identified. In addition, partial characterization of etiocholanolone (3 $\alpha$ -hydroxyetiocholan-17-one) was made. From the incubation

TABLE I  
*Steric Course of Reduction of 11-Deoxycortisol by Various Fractions of Rat Liver Homogenate*

Experiment	Tissue fraction	C <sub>21</sub> steroid		C <sub>19</sub> steroid	
		Pregnane (5 $\beta$ )	Allopregnane (5 $\alpha$ )	Etiocholane (5 $\beta$ )	Alloetiocholane (5 $\alpha$ )
A	Supernatant (78,000 $\times$ g)	3 $\beta$ , 5 $\beta$		5 $\beta$ * (etiocholanolone)	
B	Particulate fractions		3 $\alpha$ , 5 $\alpha$		5 $\alpha$
	Supernatant (78,000 $\times$ g)	3 $\alpha$ , 5 $\beta$			
	Residue (78,000 $\times$ g)		5 $\beta$ , 5 $\alpha$ †		
	" (6000 $\times$ g)		3 $\alpha$ , 5 $\alpha$		
			3 $\beta$ , 5 $\alpha$		

\* Partially characterized

† Configuration established by identification of the sodium bismuthate degradation product, epiandrosterone

tions of 11-deoxycortisol with the particulate fractions, the 5 $\alpha$  derivative androstane-3,17-dione and 3 $\alpha$ ,17 $\alpha$ -21-trihydroxyallopregnan-20-one, were isolated and identified, the identifications being based on infrared analysis, melting points, and mobility rates on paper chromatograms.

Since the yields of conversion products were low and the separation of particulate matter from the supernatant fluid had been inadequate because the homogenate was too concentrated (1 gm of liver per 0.6 ml of buffer), a second series of studies was undertaken to confirm the resolution of the  $\Delta^4$ -5 $\alpha$ - and  $\Delta^4$ -5 $\beta$ -hydrogenases. In the following group of experiments, the use of a more dilute homogenate (1 gm of liver per ml of buffer) resulted in better packing and separation of particulate matter from the supernatant fraction. The steroid concentration was decreased from 1 mg per ml of tissue preparation to 10 mg per 20 ml of tissue preparation.

### Experiment B

Rat liver (500 gm) was homogenized with 500 ml of Krebs-Ringer-phosphate buffer. After being strained through gauze, 880 ml of homogenate were obtained. Centrifugation at  $6000 \times g$  for 0.5 hour produced 475 ml of supernatant fluid and 380 ml of residue. The supernatant fluid (400 ml) was further centrifuged<sup>1</sup> at  $78,000 \times g$  for 90 minutes to yield a particulate-free supernatant fluid (340 ml) and a residue.

Three separate incubations were carried out. To this supernatant fluid, which amounted to 340 ml, 170 mg of 11-deoxycortisol,  $9.2 \times 10^{-3}$  mole of nicotinamide,  $3.1 \times 10^{-3}$  mole of potassium hexose diphosphate, and  $6.1 \times 10^{-4}$  mole of DPN were added, and the mixture was incubated at  $37^\circ$  for 2 hours. The residue from centrifugation at  $78,000 \times g$  was resuspended in buffer to a final volume of 340 ml, and 170 mg of 11-deoxycortisol,  $9.2 \times 10^{-3}$  mole of nicotinamide,  $6.12 \times 10^{-4}$  mole of DPN, and  $3.1 \times 10^{-3}$  mole of potassium hexose diphosphate were added and the mixture was incubated at  $37^\circ$  for 2 hours. The residue from centrifugation at  $6000 \times g$  was resuspended in 880 ml of buffer, and to a 120 ml aliquot of the suspension 60 mg of 11-deoxycortisol,  $3.6 \times 10^{-3}$  mole of nicotinamide,  $1.2 \times 10^{-3}$  mole of potassium hexose diphosphate, and  $2.4 \times 10^{-4}$  mole of DPN were added. This mixture was also incubated at  $37^\circ$ . Each incubation mixture was extracted as previously described (6).

*Incubation of 11-Deoxycortisol with Supernatant Fluid from Centrifugation at  $78,000 \times g$* —The extract (290 mg) was chromatographed on eight paper strips 15 cm wide in the toluene-propylene glycol system (8) for 72 hours and the runoff fraction was collected. The zone corresponding to the ring A-reduced derivative (THS) was eluted, concentrated, and dried. Crystallization from absolute ethanol yielded an initial crop of 11 mg of microcrystals with no definable form, which melted at  $206\text{--}210^\circ$ . The infrared spectrum of this compound proved to be identical to that of an authentic sample of  $3\alpha, 17\alpha, 21$ -trihydroxypregnan-20-one (m.p.  $214\text{--}216^\circ$ ). Although this confirmed the reduction of ring A to a  $5\beta$  derivative by the soluble homogenate fraction, the  $3\alpha$ -hydroxy stereoisomer was obtained here as compared to the  $3\beta$ -hydroxy isomer obtained in Experiment A.

*Incubation of 11-Deoxycortisol with Residue from Centrifugation at  $78,000 \times g$* —An aliquot of the extract (0.1 per cent of 392 mg) was applied to a paper strip 15 cm wide, chromatographed in the toluene-propylene glycol system for 72 hours, and the runoff fraction was collected. Color reactions on paper failed to locate any zones wholly characteristic of THS deriv-

<sup>1</sup> Thanks are due to the Massachusetts General Hospital and Dr. M. Hoagland for making available the ultracentrifuge and facilities for preparing the various rat liver homogenate fractions used in this study.

atives All the zones gave reactions typical of compounds with a  $\Delta^4$  ketone and  $\alpha$ -ketol side-chain systems One of these zones which corresponded in polarity to that of THS compounds was eluted and analyzed in the infrared region The analysis indicated a mixture and the carbonyl region suggested that one of the major components possessed an  $\alpha,\beta$  unsaturation Other carbonyl bands were present near the regions normally assigned to cyclopentyl carbonyl vibrations and cyclohexyl and  $\gamma$ -butyrol chain carbonyls, respectively The variation in the relative intensities of the carbonyl bands indicated that these functional groups were derived from different molecules An aliquot of the paper eluate was subjected to ultraviolet analysis and a sharp band was obtained at 240 m $\mu$  (9) was estimated from this that the  $\alpha,\beta$ -unsaturated component accounted for 40 to 50 per cent of the total weight of the dried eluate Quantitative determination on another aliquot by the Zimmermann reaction gave a value of 20 per cent of 17-ketosteroid material present in the mixture The third component in the mixture could be either a reduced steroid or some non-steroidal contaminant

A 5 mg sample of this mixture was oxidized with sodium bismuthate in order to convert the oxidizable components in the mixture to the  $C_{19}$  compounds The oxidized product (approximately 3.5 mg) was chromatographed in the ligroin-propylene glycol system (10) for 18 hours Color development with the Zimmermann reagent showed the presence of four zones, two of which were major and two minor Of the two major zones, one appeared to be a reduced  $C_{19}$  17-ketosteroid, having the mobility of epiandrosterone ( $3\beta$ -hydroxyandrostane-17-one) Infrared analysis of the material (m.p. 171–175°) and comparison of its spectrum with that of an authentic sample of epiandrosterone (m.p. 173–175°) proved that they were identical This afforded evidence for the 5 $\alpha$ -reducing type of activity in this tissue fraction, but the specific  $C_{21}$  metabolites could not be established

*Incubation of 11-Deoxycortisol with Residue from Centrifugation at 6000 g*—The extract weighing 103 mg was applied to three paper strips 10 cm wide, developed in the toluene-propylene glycol system for 72 hours, and a runoff fraction was collected Two zones corresponding to THS derivatives were developed These were eluted separately and chromatographed in the toluene-propylene glycol system for 72 hours Each component, after elution from paper and drying, was crystallized from absolute ether Infrared analysis of the two components proved that the spectrum of the first (m.p. 210–214°) was identical to that of  $3\alpha,17\alpha,21$ -trihydroxyallopregnen-3-one and the spectrum of the other (m.p. 214–218°) identical to that of  $3\beta,17\alpha,21$ -trihydroxyallopregnan-20-one (m.p. 214–216°)

## DISCUSSION

These experiments establish the fact that rat liver tissue contains two reducing systems, one of which reduces the  $\Delta^4$  group to the  $5\alpha$  stereoisomer (androsterone or allopregnanone), and the other reduces the  $\Delta^4$  group to the  $5\beta$  stereoisomer (etiocholanone or pregnanone). These enzyme systems have been named  $\Delta^4$ - $5\alpha$ -hydrogenase and  $\Delta^4$ - $5\beta$ -hydrogenase, respectively (11). The  $5\beta$ -reducing system is contained in the soluble portion of the tissue homogenate, thus confirming the observation of Tomkins and Isselbacher (7), who found the  $5\beta$  derivative of cortisone when the latter steroid was incubated with the supernatant fluid from a rat liver homogenate which had been centrifuged at  $78,000 \times g$ . The  $5\alpha$ -reducing system is associated with the particulate material (Table I).

Still unexplained are the facts that the perfusion of  $\Delta^4$ -3-ketones through rat livers and the incubation of these compounds with rat liver slices or homogenates lead to  $5\alpha$ -reduced steroids although both enzymes are present. The possibility exists that some interaction takes place which suppresses the activity of the  $\Delta^4$ - $5\beta$ -hydrogenase system. This possibility could be tested by recombining the supernatant fluid ( $5\beta$  system) produced by centrifugation at  $78,000 \times g$  with the residue ( $5\alpha$  system).

These studies also confirm our previous observations (6) that 11-deoxycortisol may be converted to 17-ketosteroids by homogenate fractions. The yields are still low and the enzyme system has not been located precisely in the various homogenate fractions.

## SUMMARY

With 11-deoxycortisol as a substrate, it has been demonstrated that rat liver contains both the  $\Delta^4$ - $5\alpha$ -hydrogenase and  $\Delta^4$ - $5\beta$ -hydrogenase systems, which yield, after incubation, the  $5\alpha$  (androsterone and allopregnanone) and  $5\beta$  (etiocholanone and pregnanone) stereoisomers. The two enzyme systems have been separated by differential centrifugation. The  $5\alpha$  system is associated with the particulate fractions, while the  $5\beta$  system is associated with the soluble supernatant fluid produced by centrifugation at  $78,000 \times g$ .

The conversion of 11-deoxycortisol to 17-ketosteroids by homogenate fractions confirms our previous finding.

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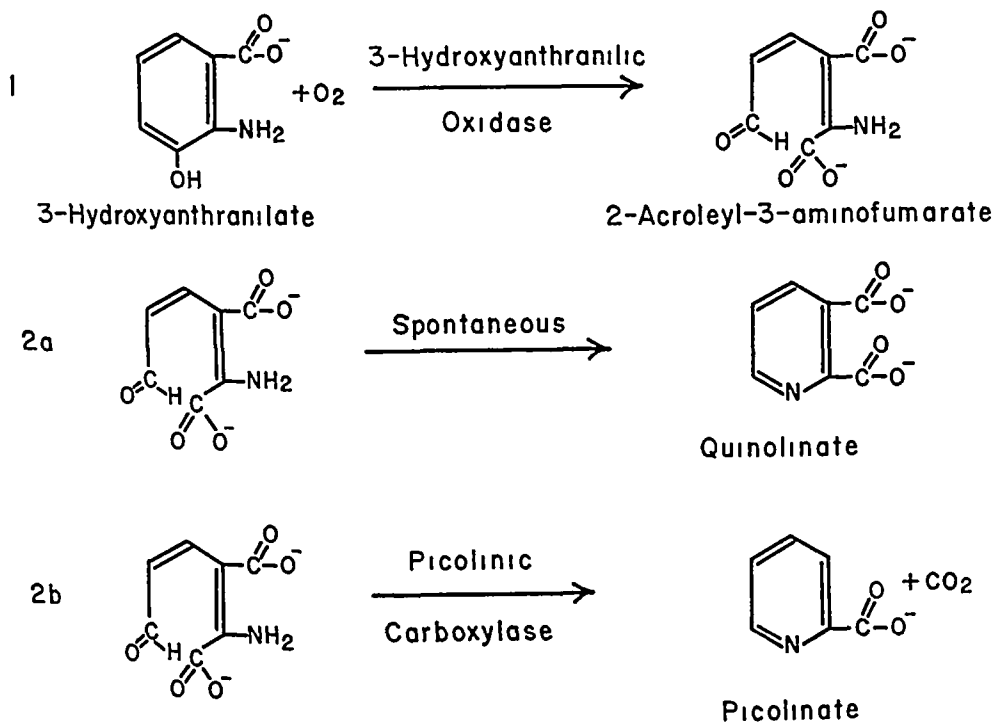
# STUDIES WITH CARBOXYL-LABELED 3-HYDROXY- ANTHRANILIC AND PICOLINIC ACIDS IN VIVO AND IN VITRO

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The formation of picolinic acid by the action of liver enzymes on 3-hydroxyanthranilic acid has been described previously (1). The reaction was postulated at that time to involve the intermediate formation of an acyclic compound that could undergo either a spontaneous cis-trans isomerization and cyclization to form quinolinic acid or through enzyme action lose a carboxyl group and cyclize to become picolinic acid. In the present work evidence is presented that the carboxyl group of 3-hydroxyanthranilic acid



is the group that is cleaved through the action of the second enzyme of the sequence, now called picolinic carboxylase. The accompanying reactions are therefore proposed to represent the metabolism of 3-hydroxyanthranilic acid by liver.



3-Hydroxyanthranilic acid is an important intermediate in tryptophan metabolism and is known to be a precursor of nicotinic acid (2). The labeled carboxyl group of 3-hydroxyanthranilic acid persists in nicotinic acid derivatives (3). In the present work it was found that most of the isotope of a small dose of carboxyl-labeled 3-hydroxyanthranilic acid given to rats appears as  $\text{CO}_2$ , whereas carboxyl-labeled nicotinic acid does not yield unlabeled  $\text{CO}_2$ . This is evidence that the decarboxylation to form picolinic acid is a major step in the metabolism of 3-hydroxyanthranilic acid in rats.

These studies involved the syntheses of carboxyl-labeled 3-hydroxyanthranilic acid and carboxyl-labeled picolinic acid. The former was prepared according to the combined sequences of D'Angeli *et al.* (4) for the preparation of 2-nitro-3-methoxybenzoic acid from *m*-methoxybenzoic acid, Ciereszko and Hankes (5) for the conversion of this product to carboxyl-labeled 2-nitro-3-methoxybenzoic acid, and Nye and Mitchell (6) for the reduction and hydrolysis to 3-hydroxyanthranilic acid. However, it was found necessary to alter the described procedures in certain steps, probably because of the reduced scale.

### Methods

*Preparation of Carboxyl- $\text{C}^{14}$ -Labeled 3-Hydroxyanthranilic Acid from 3-Methoxy-2-nitroiodobenzene*—The synthesis of carboxyl-labeled 3-hydroxyanthranilic acid devised by Ciereszko and Hankes (5) involves substitution of labeled cyanide for the halogen of 3-methoxy-2-nitroiodobenzene. The nitrile is then hydrolyzed to the corresponding carboxylic acid, which is reduced and hydrolyzed to 3-hydroxyanthranilic acid. In the exchange of cyanide for the iodine of 3-methoxy-2-nitroiodobenzene (5) very poor yields were obtained when the dry reagents were heated together, apparently because of sublimation of the iodo compound. This reaction was found to proceed smoothly with ethylene glycol as a solvent. A magnetically stirred mixture of 0.35 gm of 3-methoxy-2-nitroiodobenzene (5), 0.11 gm of  $\text{Cu}_2(\text{C}^{14}\text{N})_2$ , and 3 ml of ethylene glycol was brought quickly to boiling and refluxed briskly for 4 to 5 minutes. The cooled mixture was diluted with water, cooled to  $0^\circ$ , and filtered. The precipitate was extracted with three to four portions of boiling methanol, each extract being filtered from cuprous iodide. Dilution of the filtrate with an equal volume (about 10 ml) of water, cooling to  $0^\circ$ , and filtering gave 0.19 gm (90 per cent) of nitrile.  $\text{C}^{14}$ -labeled 3-methoxy-2-nitrobenzonitrile, m.p.  $121.5\text{--}122^\circ$ . This product was dissolved in 10 ml of water, 0.3 gm of sodium hydroxide, 2 ml of water, and 1.5 ml of alcohol were added, and the mixture was refluxed gently for 30 minutes with nitrogen bubbling through the solution. The solution was cooled, acidified with concentrated hydrochloric acid, cooled to  $0^\circ$ , and filtered to give 0.14 gm of crude acids which were crystallized at an air bath temperature of  $125\text{--}150^\circ$  (0.5 mm). Collected as thick

more volatile sublimate were 25 mg of *m*-methoxybenzoic acid, m p 104–105° (identified by elemental analysis and comparison with an authentic specimen) and then 95 mg of 3-methoxy-2-nitrobenzoic acid, m p 253–256° (decomposition). Reduction of this nitro acid by the procedure of Nye and Mitchell (6) yielded 70 mg of 2-amino-3-methoxybenzoic acid, m p 167–170°. The latter, 15 mg of red phosphorus, and 1.4 ml of 55 per cent hydriodic acid, in a sealed tube, were kept in a steam bath for 24 hours. The contents of the tube were diluted with water and the solution was filtered. The filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in 3 ml of boiling concentrated hydrochloric acid and cooled gradually (finally to 0°) to give 58 mg (73 per cent based on 2-amino-3-methoxybenzoic acid, 25 per cent based on radioactive cuprous cyanide) of carboxyl- $C^{14}$ -labeled 3-hydroxyanthranilic acid hydrochloride, m p 229° (decomposition). Additional product was obtained from the mother liquor by the addition of carrier 3-hydroxyanthranilic acid.

$C_9H_8ClNO_3$  (189.6)    Calculated, C 44.34, N 4.25, found, C 44.42, H 4.39

Picolinic acid labeled in the carboxyl group was prepared essentially as described by Murray *et al* (7) by conversion of 2-bromopyridine to 2-pyridyl lithium in ether under helium and carboxylation of the lithium compound with  $C^{14}O_2$  liberated from  $BaC^{14}O_3$ .

After adding 3 N  $HNO_3$  to the reaction mixture, the aqueous phase was put on a column of Dowex 1 acetate, 1 cm  $\times$  20 cm, which was then washed with water. The picolinic acid was eluted by a gradient method in which approximately 5 N acetic acid was added to a mixing flask containing 100 ml of water. The picolinic acid, detected by its radioactivity, appeared in a well defined fraction shortly after the eluate became acidic. The solution of picolinic acid was taken to dryness under reduced pressure and the residue was dissolved in water. This solution was chromatographed on a small column of Dowex 50 (1 cm  $\times$  12 cm) with 4 N HCl in the reservoir and 50 ml of water in the mixing flask. The fractions containing picolinic acid were dried *in vacuo*. The residue was dissolved in water and again chromatographed on Dowex 1 acetate as before. This procedure yielded fractions that exhibited the spectral characteristic of picolinic acid, and these were used without further isolation when very small quantities of highly radioactive material were desired. Other preparations were crystallized from HCl-saturated ethanol, after the addition of carrier picolinic acid, and recrystallized from ethanol-ether.

An aqueous extract of rat liver acetone powder was used as a source of 3-hydroxyanthranilic oxidase free from picolinic carboxylase (1). Picolinic carboxylase purified from guinea pig liver was used in some experiments, but it was found more convenient to use crude extracts of livers from rats.

treated with cortisone, which increase the level of picolinic carboxylate many times<sup>1</sup>. Carboxyl-labeled nicotinic acid was obtained commercially.

$C^{14}$  was determined in a windowless gas flow counter with sample size sufficiently small to cause negligible self-absorption, except in the case of the measurement of expired  $CO_2$ . In this case the  $CO_2$  was trapped in lithium hydroxide, which was diluted to about 0.001 M before plating. The self-absorption of 0.05 ml aliquots of this solution was of the order of 10 to 20 per cent.

### Results

*Enzymatic Formation of  $CO_2$  from 3-Hydroxyanthranilic Acid*— $CO_2$  liberated from carboxyl-labeled 3-hydroxyanthranilic acid was determined in systems containing both 3-hydroxyanthranilic oxidase and picolinic carboxylase and in controls with only the oxidase present. The incubation was carried out in Warburg vessels with two side arms and a center well. 0.2 ml of 1 N LiOH was placed in the center well. The main chamber contained 100  $\mu$ moles of pyrophosphate buffer, pH 9.4, enzyme, and water to give a total volume of 4.0 ml. One side arm contained 25  $\gamma$  of carboxyl-labeled 3-hydroxyanthranilic acid hydrochloride in 0.1 ml; this solution contained 15,800 c.p.m. In one series the other side arm contained 0.1 ml of 0.5 M malic acid, sufficient to bring the pH of the vessel contents to 4 to facilitate liberation of  $CO_2$ . The enzyme was either oxidase alone, 0.2 ml of 1st liver acetone powder extract (1:10), or oxidase plus carboxylase, 0.5 ml of an extract of cortisone-treated rat liver. The acetone powder extract contained sufficient oxidase to consume all of the substrate within 2 minutes at 25°. The fresh liver extract contained somewhat more of the oxidase and sufficient carboxylase to remove all of the intermediate within 3 minutes at 25°. The vessels were sealed with greased glass stoppers and the substrate was tipped in immediately. The vessels were placed in an incubator at 37° and shaken for 3 hours. The malic acid was then tipped in, and the incubation was continued for about 10 minutes. The lithium hydroxide was then removed with a pipette and the center well was rinsed with water. The combined alkali and rinses from each vessel were diluted to 5.0 ml and 0.05 ml aliquots were counted. Aliquots of the incubation mixtures were also counted directly.

The results shown in Table I demonstrate the release of labeled  $C^{14}$  when the picolinic-forming enzyme is present in addition to 3-hydroxyanthranilic oxidase. It may be noted that  $CO_2$  was readily distilled from alkali from an incubation mixture near pH 9, since no difference was found in experiments in which the pH remained near 9 and in those in which it was added.

<sup>1</sup> The role of cortisone and other hormones in controlling the level of this enzyme will be discussed in a separate publication.

*Experiments in Vivo, Picolinic Acid Metabolism*—82  $\gamma$  of picolinic acid containing 520,000 c p m were injected intraperitoneally into a rat. The urine was collected under toluene. Over half of the isotope was excreted into the urine within 6 hours, and almost all could be accounted for in 24 hours. No label was detected in the  $\text{CO}_2$ . Paper chromatography in "formix" (1) and paper electrophoresis at pH 4.5 showed all of the radioactivity to move as a single component, different from picolinic acid. In a separate experiment in which unlabeled picolinic acid was given to twenty-eight rats and the labeled urinary compound served as a tracer, the excreted product was isolated and identified as the glycine conjugate, picolinuric acid, which had been described previously as a urinary excretion product of rabbits, dogs, and frogs given picolinic acid (8). The product was

TABLE I

*Distribution of  $\text{C}^{14}$  after Enzymatic Degradation of Labeled 3-Hydroxyanthranilate*

	Experiment 1				Experiment 2	
	Oxidase		Oxidase + carboxylase		Oxidase	Oxidase + carboxylase
	Acidified	Neutral	Acidified	Neutral		
$\text{CO}_2$	0	0	12,000	12,500	0	11,700
Residue	11,700	15,300	1,860	1,800	12,500	1,680

purified by chromatography on Dowex 1 acetate, by using the same general method described above for the purification of picolinic acid. The radioactive fractions were evaporated to dryness and crystallized from 95 per cent ethanol. The spectrum showed an absorption maximum at 266  $m\mu$ , compared with the peak at 264  $m\mu$  characteristic of picolinic acid (1). Elementary analysis for C, H, and N gave results consistent with picolinuric

Calculated, C 53.88, H 4.42, N 15.54, found, C 53.33, H 4.81, N 15.49

acid. On hydrolysis with 6 N HCl for 16 hours the spectrum of picolinic acid appeared and an equivalent amount of glycine was produced.<sup>2</sup>

*3-Hydroxyanthranilic Acid Metabolism*—300  $\gamma$  of 3-hydroxyanthranilic hydrochloride containing 682,000 c p m were injected intraperitoneally into rats placed in glass metabolism cages. Air was drawn through the cage with a slight vacuum and  $\text{CO}_2$  was trapped in wash bottles containing 200 ml of 1 M LiOH. Parallel experiments were carried out with carboxyl-labeled nicotinic acid, 28  $\gamma$  containing 420,000 c p m were used.

No isotope was detected in the  $\text{CO}_2$  obtained from animals given labeled

<sup>2</sup> We are indebted to Dr. J. Rabinowitz of this Institute for the glycine determination.

nicotinic acid. In contrast, in 24 hours approximately 90 per cent of the label of 3-hydroxyanthranilic acid was found in expired  $\text{CO}_2$ .

The 24 hour urine from rats given 3-hydroxyanthranilic acid contained 43,500 c.p.m. or 6.4 per cent of the administered dose. Preliminary paper chromatograms show only one radioactive compound, but minor components could easily have escaped detection. The administered radioactive nicotinic acid was largely retained by the rat, as only 27,100 c.p.m. or 6.5 per cent, was found in the urine.

### DISCUSSION

If the quantities of 3-hydroxyanthranilic acid administered to rats were absorbed within a few hours, the amount reaching the liver would greatly exceed the amount presumably formed from tryptophan during that period. This comparison is based on the excretion of picolinic acid by the normal rat (unpublished experiments, A. H. M.), in which several hundred  $\gamma$  per day appeared in the urine. The metabolism of the labeled compound, therefore, appears to be an indication of the normal process in the intact organism. The conversion of the bulk of the labeled compound to  $\text{CO}_2$  is evidence that most of the 3-hydroxyanthranilic acid formed *in vivo* is similarly oxidized and decarboxylated.<sup>3</sup> The decarboxylation probably does not involve the other known products of 3-hydroxyanthranilic acid metabolism, quinolinic acid (10) and nicotinic acid. The latter has been found not to give rise to  $\text{CO}_2$  when administered in the quantities used in these experiments and only small amounts of  $\text{CO}_2$  were found by others when larger doses were given (11). Rats have been found to excrete the bulk of administered quinolinic acid unchanged (12), and quinolinic is not decarboxylated by liver enzymes that form picolinic acid from 3-hydroxyanthranilic acid (1). The previously reported formation of quinolinic acid from 3-hydroxyanthranilic acid *in vivo* (10) is a reflection of the spontaneous cyclization of the intermediate in the presence of large amounts of substrate.

The reaction mechanism previously proposed for the formation of picolinic acid is supported by the release of labeled  $\text{CO}_2$  by the combined action of the oxidase and the carboxylase. The structure of the intermediate oxidation product, of course, cannot be established conclusively on the basis of available information, but, since it is at the oxidation level of quinolinic acid (1), the action of picolinic carboxylase can only be a simple non-oxidative decarboxylation. The physiological importance of this

<sup>3</sup> Hanks and Henderson (9) have recently reported experiments in which carbon-14 labeled 3-hydroxyanthranilic acid was administered to rats. In these experiments larger doses than those used in the experiments reported in this paper were administered and converted to  $\text{CO}_2$ .

zyme remains obscure, since picolinic acid is retained to a very limited extent, if at all, by the intact animal

#### SUMMARY

Picolinic acid is formed by a decarboxylation of the oxidation product of 3-hydroxyanthranilic acid in which the original carboxyl group of the substrate is removed. This reaction appears to proceed at a sufficient rate *in vivo* to cause most of parenterally administered carboxyl-labeled 3-hydroxyanthranilic acid to yield labeled  $\text{CO}_2$ . Picolinic acid labeled with  $\text{C}^{14}$  has been found to be excreted essentially quantitatively as its glycine conjugate by rats.

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# PURIFICATION AND PROPERTIES OF PROCARBOXYPEPTIDASE\*

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In 1935 Anson crystallized carboxypeptidase from autolyzed beef pancreas glands (2) and reported that fresh pancreas did not contain the active enzyme, but an inactive precursor, procarboxypeptidase. The precursor was partially purified and found to be activated by trypsin (3). In contrast to the pancreatic enzymes trypsin and chymotrypsin, which are prepared directly from their purified precursors, Anson's procedure of isolating carboxypeptidase from autolyzed tissue has persisted (4). While many of the chemical and enzymatic properties of carboxypeptidase are known, little is known of the relationship of this crystalline enzyme to its precursor.

Preliminary to a study of the procarboxypeptidase system, purification of the zymogen was undertaken. Albrecht, in 1954, noted that procarboxypeptidase was stable to organic solvents (5). By successive fractionations with acetone and ammonium sulfate, she achieved preparations of about 80 per cent purity and studied their conversion to carboxypeptidase. More recently it has been seen that the yields, as well as the ease and reproducibility of preparations are improved by using defatted powders of beef pancreas as starting material.<sup>1</sup> A procedure has been developed which yields procarboxypeptidase of 95 per cent homogeneity. The properties of the purified zymogen and its relationship to Anson's crystalline carboxypeptidase are described in this paper.

## EXPERIMENTAL

*Assay System and Definition of Unit*—The increase in carboxypeptidase activity upon incubation with trypsin was used as a measure of procarboxypeptidase content. Standard activation mixtures, containing 0.1 mg. of protein N per ml. and 0.01 mg. of trypsin N per ml. in 0.04 M phosphate buffer, pH 7.8, were incubated at 37°. At intervals, aliquots were with-

\* A preliminary report of this work was presented before the Forty-seventh annual meeting of the American Society of Biological Chemists, Atlantic City, New Jersey, April 16-20, 1956 (1).

<sup>1</sup> The powders were prepared in this laboratory or supplied through the courtesy of Eli Lilly and Company.



drawn and assayed for carboxypeptidase activity, with 0.02 M carbobenzyloxycyl-L-phenylalanine<sup>2</sup> as substrate in a buffer, pH 7.5, containing 0.05 M Na Veronal-0.1 M NaCl. The enzyme was diluted with the same buffer and incubated at 25° with substrate. The amount of phenylalanine released from the peptide was measured by its ninhydrin color (6).

Units of activity are arbitrarily defined as  $1000 \times k'$ , in which  $k'$  is the first order rate constant, calculated from decimal logarithms. Specific activities of the zymogen are expressed as the number of carboxypeptidase units obtained upon activation of 1 mg of precursor protein.

*Preparation of Extracts*—It was found in preliminary experiments that aqueous extracts of the acetone powder contained as much procarboxypeptidase as did saline extracts, and at a higher specific activity. According

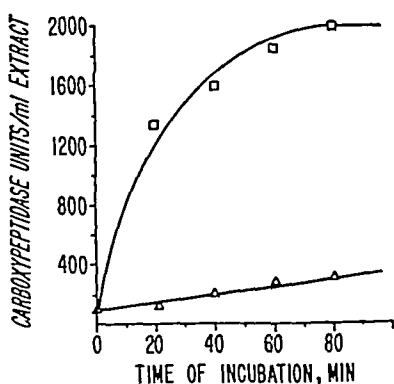


FIG. 1. The effect on carboxypeptidase activity of incubation of water extract of acetone powder of beef pancreas glands with (□) and without (Δ) added trypsin. The conditions of assay and definition of units are given in the text.

the powder was extracted with water (20 ml per gm of powder) for 24 hours, with stirring, at 5°. The suspension was centrifuged (30 minutes, 25,000  $\times g$ ), and yielded approximately 18 ml of extract per gm of starting material. Extracts prepared in this way had initially a very low level of carboxypeptidase activity when tested toward CGP. As shown in Fig. 1, incubation of the extract at pH 7.8 without added enzyme effected only a slight increase in carboxypeptidase activity, whereas in the presence of added trypsin, the activity increased 20-fold.

### Procedure of Purification

*Preparation of Acetone Powders*—Whole frozen beef pancreas glands were obtained from the slaughterhouse. The frozen glands were allowed to remain overnight in the cold room (4°) to effect partial thawing and subsequent operations were carried out in the cold room. The glands were

<sup>2</sup> The following abbreviations are used: CGP for carbobenzyloxycyl-L-phenylalanine, TCA for trichloroacetic acid.

trimmed of gross fat and connective tissue and passed twice through a chilled meat grinder. The ground tissue was extracted with 2 volumes of cold acetone, with stirring, for 6 to 8 hours. The suspension was filtered through a Buchner funnel, and as much fluid was expressed as possible. The residue was then reextracted successively twice with 2 volumes of acetone, once with 2 volumes of acetone-ether (1:1), and once with 2 volumes of ether. The defatted tissue was spread out on paper at room temperature (22°) to dry, and finally was desiccated over silica gel. 1 gm of dried acetone powder is equivalent to about 5 gm of ground pancreas.

All subsequent operations were carried out at 0° unless otherwise specified.

*Ammonium Sulfate Fractionation*—The pH of the extract (usually about 6.3) was adjusted to 7.2 by the addition of 1 N NaOH (about 0.4 ml of base per 100 ml of extract). The extract was stirred during the gradual addition of solid ammonium sulfate (27.5 gm per 100 ml to achieve 0.39 saturation). A pH of 7.2 to 7.4 was maintained throughout the fractionation by the addition of NaOH. The suspension was stirred for 30 minutes and centrifuged (30 minutes, 25,000  $\times g$ ). The precipitate was dissolved in 0.04 M phosphate buffer, pH 7.4 (about 10 per cent of the initial volume of the extract), and dialyzed overnight *versus* the same buffer.

After dialysis, the protein concentration was adjusted to 2 per cent. A saturated solution of ammonium sulfate (saturated at 0°, adjusted to pH 7.4) was added slowly, with stirring. About 47 ml were added per 100 ml of protein solution to achieve approximately 0.32 saturation. After equilibration the suspension was centrifuged, and the small precipitate was discarded. To each 100 ml of supernatant solution 11.5 ml of saturated ammonium sulfate were added to achieve 0.39 saturation. After 30 minutes with stirring, this suspension was centrifuged, and the precipitate was dissolved in water (about 5 per cent of the initial volume of extract) and dialyzed overnight *versus* water.

*Isoelectric Precipitations*—Procarboxypeptidase is minimally soluble in water at pH 5.2. Further purification was achieved as follows. The aqueous solution of procarboxypeptidase was diluted to a protein concentration of 2 per cent. Acetic acid (0.1 M) was added dropwise, and with stirring, until a pH of 5.2 was reached. The suspension was stirred for 30 minutes and centrifuged. The precipitate was resuspended in the same volume of water, 0.2 M Ba(OH)<sub>2</sub> was added to pH 6.0, and the suspension was stirred at pH 6 for several hours. Procarboxypeptidase is extracted in this way while several of the contaminating proteins remain insoluble. After centrifugation, the proteins of the supernatant fluid were usually reprecipitated at pH 5.2 and once again extracted at pH 6.0. Preparations of procarboxypeptidase were routinely stored in the cold as a lyophilized powder.

Table I presents a typical protocol, which shows recoveries, enrichment,

and the electrophoretic homogeneity at each stage. By extrapolation of these data it is estimated that procarboxypeptidase of 100 per cent homogeneity would have a specific activity of 1000.

It will be noted that about 35,000 units of potential carboxypeptidase activity were extracted from each gm of powder ( $\cong$  5 gm of fresh tissue). This value compares favorably to the total activity extracted directly from fresh tissue, *viz.*, 21,000 units per 5 gm and indicates that exposure to the organic solvents was in no way deleterious to the enzyme.

### *Properties of Procarboxypeptidase*

Preparations of procarboxypeptidase obtained by the procedure described in the preceding section were approximately 95 per cent homogeneous.

TABLE I  
*Protocol of Purification of Procarboxypeptidase*

100 gm of powder were used

Stage in purification	Total units*	Total protein	Specific activity	Electrophoretic homogeneity
		mg	units per mg	per cent
1 Water extract	3,480,000	21,750	169	91
2 1st $(\text{NH}_4)_2\text{SO}_4$ ppt (0.0–0.45)	2,700,000	4,675	557	95
3 2nd $(\text{NH}_4)_2\text{SO}_4$ ppt (0.32–0.39)	1,253,000	1,702	736	95
4 Isoelectric ppt, pH 5.2, extracted with $\text{Ba}(\text{OH})_2$ , pH 6.0	941,000	1,032	911	95

\* Units =  $k' \times 1000$ , in which  $k' = 1/t(\log a/a - x)$

ous. The physicochemical properties of the purified zymogen were examined and compared with those of Anson's crystalline carboxypeptidase. The results are summarized in Table II.

**Electrophoretic Analysis.**—Electrophoresis was carried out in monovalent buffers of ionic strength 0.2 over a pH range of 4.5 to 7.5 in a Spinco model H electrophoretic apparatus. Fig. 2 shows a typical electrophoretic pattern. The protein used in these experiments was 97 per cent homogeneous. The pH-mobility curves of procarboxypeptidase and of Anson's crystalline carboxypeptidase are shown in Fig. 3. It will be seen that procarboxypeptidase is the more acidic protein. Under the same conditions of electrophoresis, the isoelectric point of the zymogen is about 2 pH units below that of crystalline carboxypeptidase. That the observed isoelectric point of procarboxypeptidase is due in part to the binding of buffer ions is indicated by the fact that in aqueous solutions the pH of minimal solubility is 5.2. It was noted also that a solution of procarboxypeptidase

TABLE II

*Properties of Procarboxypeptidase and of Crystalline Carboxypeptidase*

Property	Procarboxypeptidase	Crystalline carboxypeptidase
Isoelectric point, univalent buffer, $\Gamma/2 = 0.2$	<pH 4.5	pH 6.0
$S_{20}^{25}$	5.87 S	3.07 S
Molecular weight	96,000 (light scattering) 96,000 (sedimentation equilibrium)	34,000
% nitrogen	15.9	15.4
$E_{280}^{1\%}$	19	23
$\epsilon$	$18.2 \times 10^4$	$7.9 \times 10^4$
Specific activity (0.02 M CGP)	1000 units per mg	3000 units per mg

The properties of procarboxypeptidase were determined as described in the text. The properties of crystalline carboxypeptidase were described elsewhere (7-9).

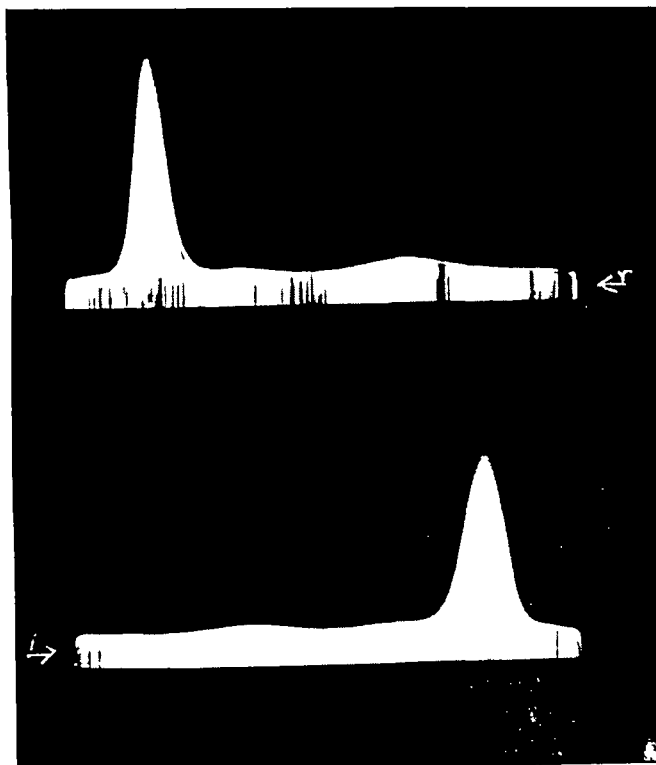


FIG. 2. Electrophoretic pattern (300 minutes at 3.36 volts per cm) of procarboxypeptidase in cacodylate-NaCl buffer, pH 6.75, of ionic strength 0.2. Upper photograph, ascending boundary; lower photograph, descending boundary.

tained a stable pH of 5.2 when deionized by passage through a mixed bed ion exchange column (1:2 mixture of IR-120, IR-400, 30 mesh)

**Sedimentation Analysis**—Solutions of procarboxypeptidase were analyzed in the ultracentrifuge (Spinco model E, at 20°, in the standard cell) in 0.05 M glycyl-L-histidyl-L-glutamate-NaCl buffers of ionic strength 0.2, pH 6.75. Sedimentation constants were obtained over the concentration range of 0.1 to 1.2 per cent.

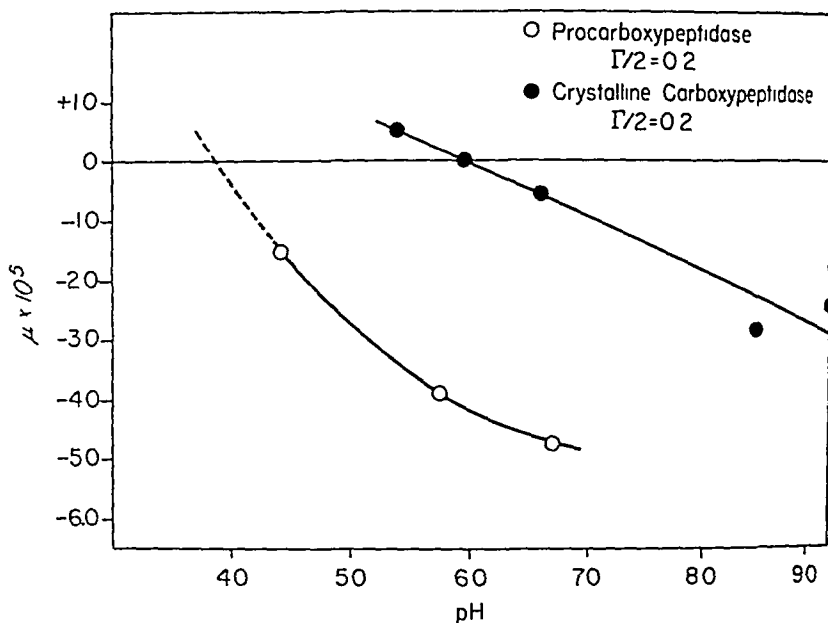


FIG. 3. pH mobility curves of procarboxypeptidase (O) and of crystalline carboxypeptidase (●) (7). Monovalent buffers of ionic strength 0.2 were used in both series.

protein. Extrapolation of these values to infinite dilution gives the value  $s_{20,w} = 5.8$  S for procarboxypeptidase. The sedimentation constant of Anson's crystalline carboxypeptidase is reported to be 3.08 (8).

**Light scattering measurements**<sup>3</sup> indicate that the molecular weight of the purified zymogen is 96,000. This value was confirmed in sedimentation equilibrium experiments and is almost 3 times the molecular weight of Anson's enzyme (34,000). Correspondingly, specific activities based on precursor protein are about one-third those of the crystalline carboxypeptidase. On a molar turnover basis, the potential activity of the zymogen is about equal to the activity of crystalline carboxypeptidase.

The **absorption spectrum** of procarboxypeptidase is that of a typical protein, with a maximal extinction at 280 m $\mu$  and a ratio of extinction at 260 and 280 m $\mu$ , respectively, of 0.58. The extinction coefficient of the

<sup>3</sup> Unpublished experiments performed by Dr. Joseph Kraut, Department of Chemistry, University of Washington. Details of the determination of the molecular weight of procarboxypeptidase will be presented in a separate publication.

zymogen was obtained by dry weight determinations on samples of known optical density  $E_{280}^{1\%}$  was found to be 19. Hence the molar extinction coefficient of procarboxypeptidase (molecular weight = 96,000) is  $1.82 \times 10^5$ . Kjeldahl nitrogen determinations were related to the dry weight of the protein to give a nitrogen content of 15.9 per cent.

### Activation Reaction

The activation of procarboxypeptidase was followed in the ultracentrifuge. Solutions of the zymogen (0.2 mg of N per ml) were incubated

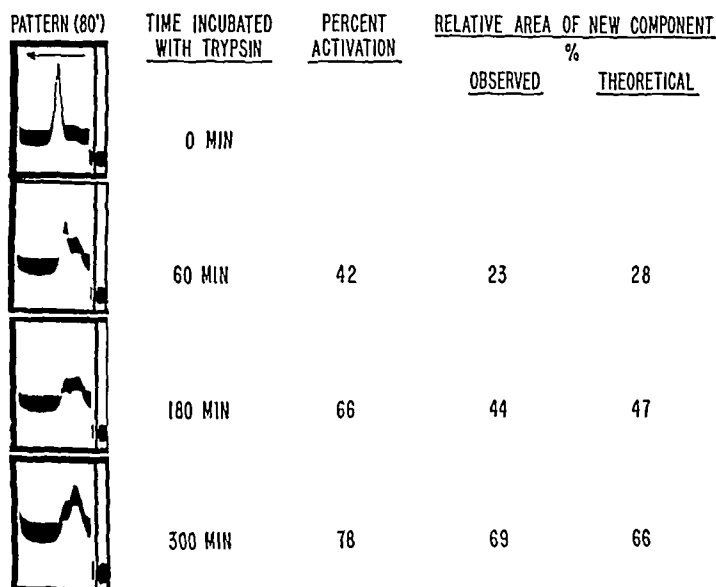


FIG. 4. Sedimentation patterns of partial activation mixtures of procarboxypeptidase. Experimental details are described in the text.

with trypsin (0.008 mg of N per ml) at pH 7.8, 37°, and at intervals aliquots were withdrawn for enzymatic assay. Concurrently, soy bean trypsin inhibitor was added to portions of the activation mixture to arrest further action by trypsin. The latter portions then were dialyzed, lyophilized, and, after equilibration with buffer (0.04 M  $\text{PO}_4$ -0.1 M LiCl, pH 7.4), subjected to sedimentation analysis. Fig. 4 presents the sedimentation patterns of partial activation mixtures, corresponding to 42, 66, and 78 per cent activation, respectively. It will be noted that, during incubation with trypsin, procarboxypeptidase disappears and a single new protein component, with a lower sedimentation constant ( $s_{20,w} = 3.0$  S at a protein concentration of 0.8 per cent), appears. The relative area of the new protein component is at all times less than would be expected from the degree of activation. In control experiments, solutions of the same preparation

of zymogen were incubated with trypsin to which an equal weight of bean trypsin inhibitor had been added. The sedimentation pattern of such mixtures were unchanged during 240 minutes of incubation.

While there might be several explanations for the appearance of a new protein component with a lower sedimentation constant, the following experiments have provided evidence that the new component is, in fact, a smaller molecule, carrying all of the enzymatic activity, and that the residual inert moiety of the zymogen can be accounted for as dialyzable fragments soluble in 15 per cent trichloroacetic acid (TCA). The course of activation of procarboxypeptidase was followed simultaneously by enzymatic assay and by the appearance of TCA-soluble material. For the latter, aliquots were withdrawn and added to an equal volume of cold 5

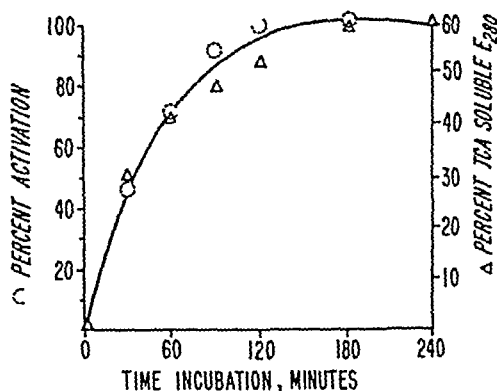


FIG. 5. Correlation of the per cent of activation of procarboxypeptidase (O) and the appearance of TCA-soluble material (Δ) during incubation with trypsin. Experimental details are described in the text.

per cent TCA. After 30 minutes at 0°, the precipitate was centrifuged and the extinction of the supernatant solution was measured at 280 mμ. As shown in Fig. 5, the data followed a single curve, when the scale was adjusted so as to equate 100 per cent activation to the condition in which 57 per cent of the total extinction of the precursor was soluble in TCA. It is significant that the fraction of the total extinction which remains precipitable by TCA (0.43) is precisely the ratio of molar extinction coefficients of crystalline carboxypeptidase and of the purified zymogen. With the net loss in protein material during activation is taken into account, the observed relative areas of the new protein component shown in Fig. 4 agree well with the predicted value.

#### *Product of Activation*

The activation of procarboxypeptidase was carried out on a larger scale and the enzymatically active product was isolated and crystallized. The crystalline pattern of carboxypeptidase prepared from procarboxypeptidase

is shown in Fig 6. Some of the molecular properties of this enzyme are presented in Table III, along with those of carboxypeptidase isolated from autolyzed pancreas glands by the procedure of Anson. The sedimentation



FIG 6 Crystalline habit of carboxypeptidase prepared from purified procarboxypeptidase

TABLE III

*Properties of Carboxypeptidase Prepared from Purified Procarboxypeptidase*

Property	Carboxypeptidase prepared	
	According to Anson	From purified procarboxypeptidase
Crystalline habit	Boat shaped	Crescent shaped
$s_{20, w}$	3.07 S	3.06 S
$\mu^*$ in $\text{PO}_4\text{-LiCl}$ buffers, pH 7.4, $\Gamma/2 = 0.2$	$-1.83 \times 10^{-5}$	$-1.44 \times 10^{-5}$
Specific activity	3000 units per mg	3000 units per mg

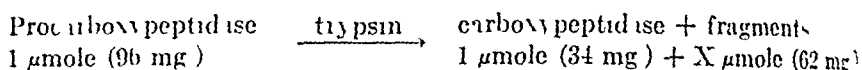
\*  $\mu$  = electrophoretic mobility

constants of the two enzymes are identical, whereas the significance of the slight difference in electrophoretic mobilities is not yet known. It will be noted, however, that the specific activity of the enzyme prepared from procarboxypeptidase is 3 times higher than that based on precursor protein and is exactly equal to the specific activity of Anson's enzyme.



## DISCUSSION

The conversion of procarboxypeptidase to carboxypeptidase can be summarized at present by the equation



The reaction is trypsin-dependent, as neither activation nor the molecular changes occur when procarboxypeptidase is incubated with soy bean inhibited trypsin. Trypsin apparently catalyzes both the activation and the conversion to the smaller molecule. All of the potential activity of the precursor (96,000 units per  $\mu\text{mole}$ ) can be accounted for in the isolated carboxypeptidase (102,000 units per  $\mu\text{mole}$ ).

The possibility that contaminant enzymes in procarboxypeptidase preparations might be involved in the reaction was minimized by the observation that procarboxypeptidase, isolated electrophoretically (hence free from contaminants other than any with the same mobility), showed the same behavior when incubated with trypsin as did preparations prior to electrophoresis. Furthermore, the reaction goes to completion in the presence of  $\beta$ -phenylpropionate (0.1 M) which would inhibit either chymotryptic activity or any autolytic action by the carboxypeptidase formed.

The activation reaction proceeds in an "all or none" manner. It has not been possible to separate the profound molecular degradation from the formation of active carboxypeptidase. No intermediate enzyme or zymogen forms have been detected as yet. The relationship of procarboxypeptidase to carboxypeptidase is markedly different from other zymogen-enzyme relations. Trypsin and chymotrypsin have about the same molecular weights as their respective precursors, and a limited number of peptide bonds are split during their activation (10). Much more extensive degradation accompanies the activation of procarboxypeptidase.

Several possible mechanisms may be considered for the activation process. Trypsin may, in an initial phase, split a limited number of peptide bonds, leading to a disorientation of the chain structure of the inert moiety of the precursor molecule and, consequently, to further degradation by trypsin. It would appear, from the specificity of the activating enzyme trypsin, that the non-enzymatic moiety of the precursor contains a less accessible lysyl or arginyl bonds than does carboxypeptidase.

A second possibility that may be considered is that, analogous to the activation of pepsinogen (4), procarboxypeptidase is converted by trypsin to a carboxypeptidase-inhibitor complex and that, upon dissociation from the inhibitor is digested by trypsin.

Thirdly, procarboxypeptidase, although behaving as a homogenous molecular species may, in fact, be a complex of two or more protein

tivation, in this event, might be accompanied by or even consist in tryptic digestion of the inert moiety of the complex

Experiments are in progress to test these hypotheses and to characterize further procarboxypeptidase, its relationship to carboxypeptidase, and the role of metal in the activation process

#### SUMMARY

A procedure has been developed for the preparation of procarboxypeptidase from acetone powders of beef pancreas glands. The final product is about 95 per cent homogeneous during electrophoresis and ultracentrifugation. The properties of the purified zymogen have been compared with those of crystalline carboxypeptidase.

Procarboxypeptidase is a larger molecule (molecular weight 96,000) than crystalline carboxypeptidase (molecular weight 34,000), with more acidic properties and with an isoelectric point about 2 pH units lower than that of carboxypeptidase.

Upon activation by trypsin, the purified zymogen gives rise to a smaller protein molecule, which has been crystallized. All of the potential activity of the zymogen can be accounted for in the crystalline carboxypeptidase isolated from the activation mixtures.

Our thanks are due to Dr. Eric Stein for his cooperation during the initial phase of this work and to Mr. Roger D. Wade for performing the electrophoretic and ultracentrifugal analyses. This work was supported in part by research grant No. BCH-42 from the American Cancer Society and by research grant No. C-2286 from the National Institutes of Health, Public Health Service.

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# INFLUENCE OF CATIONS ON THE INTRACELLULAR DISTRIBUTION OF RAT LIVER ARGINASE\*

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Reports from several laboratories (3-7) indicate that the arginase content of rat liver is positively correlated with the protein catabolism of the animal. The changes in the enzyme content are always initiated by parallel changes in the quantity of total liver protein and follow rather than precede the changes in protein catabolism. The phenomenon appears to represent neither merely a non-specific participation of the enzyme in the net changes of hepatic protein nor a specific adaptive response, but rather a combination of both processes. It has been interpreted by us (7) as reflecting excessive formation or deterioration of the enzyme in response to a functional overload or non-use.

This interpretation was based on the fact that the response of arginase to changes in the protein catabolism was excessive with respect to that of the total hepatic protein and of enzymes functionally unrelated to arginase. The evidence remains incomplete, however, unless it is known that arginase and the reference enzymes used have the same intracellular localization. As long as such information is lacking, the possibility cannot be excluded that changes in the relative amounts of the morphological units, of which the enzymes are among the components, may simulate a specific response of arginase to metabolic stimuli when there is only non-specific participation in a localized gain, and loss of protein not recognizable in the net protein changes of the organ.

We have used mainly rhodanese as a reference enzyme for arginase, since both enzymes are probably situated exclusively in the parenchymatous liver cells. Rhodanese is known to be localized in the mitochondria (7, 8). From reports in the literature (8, 9) and earlier studies from this laboratory, it appeared that arginase is bound mainly to nuclei and microsomes. The results of the present investigation show, however, that this distribution pattern holds only for tissue homogenates in electrolyte-free

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aqueous media. At cation concentrations approaching those prevailing in intact liver cells, arginase is not associated with particulate cell components. The response to changes in protein catabolism of suitable reference enzymes belonging to the soluble fraction of hepatic cytoplasm remains to be explored. Implications of these findings for enzyme distribution studies will be discussed.

### Methods

Male rats, 250 to 300 gm, from the colony of the Wistar Institute were fasted overnight and anesthetized with Amytal, and the livers were subjected to retrograde (10) perfusion *in situ* with cold 0.25 M sucrose solution. Perfusion and all subsequent procedures were carried out in the cold room at 4°. The excised liver was briefly minced with scissors and portions of about 2 gm each were strained through the wire screen of a small stainless steel hand press, a fresh screen being used for each portion to prevent heating up of the pulp and the resulting exposure to high pressure. 10 per cent homogenates in the sucrose medium were prepared in a modified Potter-Elvehjem (11) apparatus<sup>1</sup> equipped with plastic pestle head. A similar type of grinding vessel was used for centrifugal sedimentation and washing of the nuclear fraction, the supernatant fluids being removed by siphoning. The centrifugal fractionation of the homogenates was carried out according to the directions of Schneider and Hogeboom (12) with reference to steps, timing, and the centrifugal forces<sup>2</sup> used, except that the mitochondria were sedimented at  $12,300 \times g$  (15,600 r.p.m.)<sup>3</sup> during the two washings.

Purified nuclear fractions were prepared by gradient centrifugation in calcium-containing sucrose solution according to the procedure of Hogeboom, Schneider, and Striebig (13). Purification was checked by determining the deoxyribonucleic acid or nitrogen ratio and by microscopic inspection with phase contrast optics. The best results were achieved by filtering the homogenates first through four layers of surgical gauze, and then after through one layer of single napped flannelette (only the experiments

<sup>1</sup> Tissue grinders, Arthur H. Thomas Company, Philadelphia. In addition to standard equipment, the following items not listed were obtained: (a) straight grinding vessels, size B, 120 mm high, 30 ml capacity for sedimentation and purification of the nuclear fraction, (b) pestles with non-serrated Teflon grinding heads, (c) resuspending sediments in grinding vessels or Lusteroid centrifuge tubes, and (d) pestle with serrated Kel-F grinding head for the primary homogenization in experiments of Tables I, II, and IV.

<sup>2</sup> All relative centrifugal forces are calculated for the center of the tube.

<sup>3</sup> International refrigerated centrifuge No. PR-1, multispeed attachment, No. 296, 1 × 2½ inch Lusteroid tubes. The fluid volumes were 15 ml, corrected to 10 ml of original homogenate, for the initial sedimentation of the mitochondria and 8 ml for the two washings of the combined sediments of two 15 ml samples.

carried out with the latter procedure are recorded in Table II) The omission of straining of the liver before homogenization (14) failed to improve on the chemical and morphological properties of the preparations, but resulted in much smaller yields of nuclei

For the assay of nucleic acids, the extraction procedure of Schneider (15) was used Deoxyribonucleic acid (DNA) was determined with the diphenylamine reaction of Dische (16) in a Beckman DU spectrophotometer at 600  $m\mu$ , and ribonucleic acid (RNA) with the orcinol reaction<sup>4</sup> (17) in a Klett-Summerson colorimeter equipped with filter No 66

Determinations of total nitrogen and of arginase have been described previously (5) For the latter assay, aliquots were immediately withdrawn at each step of the fractionation procedure and diluted with the manganese sulfate-saline solution, as outlined previously The enzyme activity of these mixtures remains unchanged for storage periods in the cold up to a week

Magnesium was estimated colorimetrically, with the titan-yellow method of Heagy (18) adapted to smaller samples Readings were made in the Klett-Summerson instrument with filter No 56, as the ratio of magnesium complex absorption to blank absorption at 560  $m\mu$  was more favorable than at the absorption maximum of 540  $m\mu$  Blanks and standards contained the same quantity of sucrose as the experimental samples

### Results

*Intracellular Distribution of Arginase*—The intracellular distribution of rat liver arginase has been previously studied by Schein and Young (9) in distilled water homogenates and by Ludewig and Chanutin (8) in isotonic sucrose solution In both media, 75 to 80 per cent of the enzyme activity remained bound to particulate matter, the nuclear fraction accounting for about 35 per cent of the total activity The percentage present in the mitochondrial fraction was 31 and 15 per cent in distilled water and isotonic sucrose solution, respectively The latter value closely agrees<sup>5</sup> with those found by us (7) in mitochondria, from which the fluffy layer was not removed In contrast, the modified mitochondrial preparation used in the present study (Table I) contained but 6 per cent of the arginase activity, indicating that the higher previous values were due to contaminating microsomes Because of the known heterogeneity of the latter fraction, an experiment was performed in which two subfractions, which

<sup>4</sup> Iron reagent, 0.1 per cent ferric ammonium sulfate  $12H_2O$  in concentrated HCl Heating time, 45 minutes

<sup>5</sup> In protein-fed rats, the arginase distribution among the four fractions was 29, 13, 37, and 5 and 26, 15, 41, and 7 in resting and in 1 day-regenerating liver tissue, respectively

sedimented at  $27,000 \times g$  (30 minutes)<sup>6</sup> and  $78,000 \times g$  (60 minutes) were assayed individually. The specific arginase activity of these fractions was 53.8 and 49.5, respectively, indicating a fairly even distribution of the enzyme among microsomes of different sizes. From Table I it is evident that the relative concentration of arginase in the nuclear, mitochondrial, and microsomal fractions followed that of the nucleic acids, suggesting that the latter were instrumental in the binding of the enzyme.

TABLE I

*Distribution of Arginase, Total Nitrogen, and Nucleic Acids in Rat Liver\**

Fraction	Per cent distribution†				Relative concentration			
	Arginase	Total N	DNA	RNA	Arginase	DNA	RNA‡	Total
Homogenate (H)	100 (923)	100 (33)	100 (3.1)	100 (8.2)	1.00 (28.9)	1.00 (92.0)	1.00 (238.5)	1.00 (3.1)
Nuclei (Nw)	35	17.9	87.4	9.1	1.96	4.86	0.53	1.00
Mitochondria (Mw <sub>2</sub> )	5.8	26.9		9.4	0.22		0.20	0.07
Microsomes (P)	41.3	27.0		62.4	1.54		2.40	1.00
Supernatant fluid (S <sub>2</sub> )	2.2	22.7		12.3	0.07		0.44	0.00
Nw + Mw <sub>2</sub> + P + S <sub>2</sub>	84.3	99.7	87.4	93.2				

\* Mean values of four experiments

† ((Amount in fraction)/(amount in homogenate))  $\times$  100. For fraction H enzyme units or mg of constituent per gm of perfused liver tissue are added in parentheses. Arginase units are expressed as micromoles of urea formed per minute at pH 9.5 and 25°.

‡ Enzyme units or micrograms of constituent per mg of total nitrogen; homogenate concentrations are taken as unity. The latter are also recorded in absolute terms in parentheses.

§ The ratio RNA/DNA was 2.61 and 0.27 in H and Nw, respectively.

to the particles. The enzyme concentration in the soluble fraction was disproportionately low. However, enzyme recoveries were incomplete, a fact also noticed by Ludwig and Chanutin. The loss in enzyme activity amounted to 5 and 10 per cent after separation and washing of the nuclear and mitochondrial fractions, respectively, with the procedure used in Table I and increased slightly when additional purification steps were used.

*Arginase Activity of Isolated Nuclei*—The specific arginase activity of isolated rat liver nuclei, in contrast to that of the crude nuclear fraction, has been reported as no higher than that of the original homogenate. This holds true for nuclear preparations obtained with such diverse

<sup>6</sup> Spinocoultracentrifuge, type L, rotor No. 30, 17,500 and 30,000 r.p.m., ref.

ods as dispersion in citric acid with the Waring blender (19), grinding of tissue homogenates in isotonic sucrose medium by means of a special steel mill (20), and fractionation of dehydrated tissue by gradient centrifugation in non-aqueous media (21). Table II shows that the relative arginase concentration in nuclei purified by the comparatively mild procedure of gradient centrifugation in sucrose solutions remained as high as that of the crude nuclear fractions. It did not increase, however, as one would expect it to do if the arginase were bound only to DNA, because the relative concentration of this specific nuclear constituent increased 2.6 times during purification. This discrepancy might be due in part to the solubility of arginase in the calcium ion-containing sucrose media. An additional possibility is the presence in the crude nuclear fraction of microsomal elements of high specific arginase activity which were removed during purification.

TABLE II  
*Arginase Activity and Composition of Purified Liver Nuclei\**

Denotation	Arginase	Total N	DNA	RNA†
Per Cent amount in unfiltered homogenate	9.3	4.5	57.3	3.2
Concentration, units or $\gamma$ per mg N	57.1		1284.5	192.8
Relative concentration‡	2.1		12.8	0.6

\* Mean values of two experiments

† RNA/DNA = 0.15

‡ With reference to concentrations in unfiltered homogenate

Even the purified nuclear fractions were not homogeneous microscopically. The most conspicuous contaminations were collapsed cell membranes and some larger structures, which appeared to be cytoplasmic aggregates and might have contained microsomal material. Since attempts at separating the contaminants from the nuclei by filtration or fractional centrifugation were unsuccessful, their arginase activity remains unknown.

*Elution of Arginase by Cations*—Since manganese ions preserve the activity of arginase for extended storage periods, it was thought possible to improve on the enzyme recoveries by adding 5 mmoles per liter of manganese sulfate to the supernatant fluid of the crude nuclear fraction. This procedure, however, led to the removal of arginase from the particles. Hence, a more detailed investigation of the solubility of nuclear and microsomal arginases in the presence of electrolytes was undertaken.

In the experiments listed in Table III, the crude (once washed) nuclear fraction,  $N_w$ , was washed two additional times with either electrolyte-free or electrolyte-containing isotonic sucrose solution. The resuspended nuclear sediment and the two combined washings are designated as  $N_{w3}$ .



and  $\text{HS}_2$ , respectively. Since the sulfates and chlorides of manganese, magnesium and sodium gave identical results, the data obtained with both types of salts have been pooled and only the cations under study recorded. It is evident that divalent cations at a concentration of 10 meq per liter extracted 80 to 90 per cent of the arginase activity, but only 10 to

TABLE III

*Extraction of Arginase and Nitrogen from Nuclear Fraction of Rat Liver by Cation-Containing Isotonic Sucrose Solution*

Cation		No of experiments	Per cent extracted*		Relative specific arginase activity†		
Type	Concentration meq per l		Arginase	Nitrogen	$\text{Nw}$	$\text{Nw}_3$	$\text{HS}_2$
$\text{Na}^+$	0.0	2	29.3 (26.8, 31.8)	52.2 (52.0, 52.3)	1.47 (1.36, 1.56)	2.10 (2.09, 2.12)	0.86 (0.71, 1.02)
	5.0	1	20.0	45.0	1.64	2.26	0.56
	10.0	3	10.8 (7.5-12.8)	37.4 (30.4-44.0)	1.59 (1.26-2.06)	2.26 (1.99-2.74)	0.43 (0.34-0.48)
	10.0	1	12.7	38.4	1.64	2.26	0.52
$\text{Ca}^{++}$	0.36	1	28.3	56.1	1.54	2.52	0.78
	3.6	3	20.9 (13.0-27.4)	22.4 (13.7-30.6)	1.39 (1.38-1.40)	1.43 (1.40-1.46)	1.27 (1.24-1.30)
$\text{Mg}^{++}$	10.0	3	79.1 (78.0-81.0)	10.8 (6.5-15.0)	1.46 (1.25-1.57)	0.33 (0.32-0.39)	12.00 (8.80-18.4)
	1.0	1	14.0	43.0	1.93	2.96	0.60
	10.0	2	85.8 (83.0, 88.6)	22.3 (22.0, 22.6)	1.96 (1.92, 1.99)	0.36 (0.28, 0.44)	7.60 (7.60, 7.60)
	2.5	1	15.4	10.9	2.65	2.50	0.37
$\text{Mn}^{++}$	5.0	2	69.5 (66.4, 72.6)	11.2 (10.0, 12.3)	2.24 (2.20, 2.28)	0.78 (0.69, 0.86)	14.00 (13.1, 14.0)
	10.0	3	92.2 (88.6-94.0)	17.4 (14.8-22.5)	2.09 (1.86-2.31)	0.20 (0.17-0.25)	10.40 (8.8-14.4)

\*  $100 \times (\text{amount in } \text{HS}_2) / (\text{amount in } \text{Nw}_3 + \text{HS}_2)$

†  $(\text{Units per mg of N in fraction}) / (\text{units per mg of N in original homogenate})$   
Most of the  $\text{Nw}$  values are computed from the measured  $\text{Nw}_3$  and  $\text{HS}_2$  values.

20 per cent of the nitrogen of the nuclear fraction. The water-clear extracts exhibited up to 18 times the specific activity of the original homogenate. In contrast, washing with plain sucrose solution removed more nitrogen than arginase, the specific activity of  $\text{Nw}_3$  exceeding that of  $\text{Nw}$ . Sucrose solutions, supplemented with monovalent cations up to 10 meq per liter or with divalent cations below 2.5 meq per liter, behaved like plain sucrose solution. In connection with the identical specific arginase activity of crude and purified nuclear fractions, it should be noticed that

the presence of 3.6 m eq per liter of calcium ions, the concentration used in preparing homogenates for the isolation of nuclei, caused some elution of the enzyme, as indicated by the fact that the specific activities of fractions NW, NW<sub>3</sub>, and HS<sub>2</sub> were almost identical

TABLE IV  
*Extraction of Microsome-Bound Arginase by Cations*

Cation*		No of experiments	Per cent extracted			Arginase relative specific activity†		
Type	Concentration		Arginase	Nitrogen	RNA	P	Pw	S <sub>2</sub>
None	m eq per l	5	7.1 (2.6-12.5)	9.6 (7.2-13.2)	10.0‡ (7.6-13.6)	1.51 (1.37-1.70)	1.55 (1.39-1.65)	0.87 (0.05-2.12)
K <sup>+</sup>	10	1	6.5	4.6	0.1	1.42	1.40	2.02
"	77	1	90.9	14.7	0.0	1.45	0.15	8.94
"	155	2	92.7 (92.4, 93.0)	20.2 (19.4, 21.0)	0.1§	1.71 (1.65, 1.77)	0.16 (0.14, 0.17)	7.86 (7.25, 8.47)
Ca <sup>++</sup>	10	1	79.5	9.9	1.3	1.49	0.34	12.0
Mg <sup>++</sup>	10	1	84.9	9.8	1.1	1.71	0.29	15.1
"	10	2	86.8 (84.5, 89.0)	10.7 (10.0, 11.4)		1.74 (1.65, 1.82)	0.26 (0.23, 0.29)	14.1 (12.4, 15.8)

\* Isotonic solutions of the chlorides (0.155 M KCl, 0.1 M divalent cations) were diluted to the desired concentration with 0.25 M sucrose solution

† P, Pw, and S<sub>2</sub> refer to unwashed microsomes, once washed microsomes, and the corresponding supernatant solution, respectively. Other denotations as in Table III. The microsomes were resedimented by centrifugation at 40,000 r.p.m. (105,000 × g) for 30 minutes

‡ Three experiments in which the extraction of arginase averaged 10 per cent

§ One experiment

The data in Table IV demonstrate that 80 to 90 per cent of the microsome-bound arginase was eluted by a single extraction with sucrose solution containing 10 m eq per liter of divalent cations. It is of interest to note that 90 per cent elution was also achieved when the potassium ion concentration was raised to 77 m eq per liter, *i.e.*, it was approaching the intracellular level. The only difference was that more nitrogen than in the experiments with divalent cations was extracted. Plain sucrose solution extracted small yet variable amounts of arginase. In addition, the extracts contained roughly 10 per cent of the microsomal RNA, whereas in electrolyte-containing media a maximum of 1.3 per cent was eluted. It is

possible that, in the absence of electrolytes, variable amounts of arginase containing degradation products of the microsomes remained in solution

*Intracellular Distribution of Magnesium Ions*—In view of the elution of particle-bound arginase by added divalent cations, information was desirable on the quantity and distribution of the cations originally present in the liver preparation. Particularly, it was thought that bound cation might hinder binding of arginase by particles, and thereby determine the distribution pattern of the enzyme. Since magnesium represents the quantitatively most important intracellular divalent cation, its distribution in electrolyte-free isotonic sucrose solution was explored (Table V). While magnesium, like arginase, was largely particle-bound, its concentration

TABLE V  
*Intracellular Distribution of Magnesium in Rat Liver\**

Designation	H	Nw	Mw <sub>2</sub>	Pw	Total part of bound Mg
Distribution, %	100†	15.6 (9.9–19.6)	25.6 (24.2–27.2)	36.4 (35.0–38.8)	77.5 (72.9–80.1)
Relative concentration	1.00‡	0.87 (0.52–1.06)	0.94 (0.92–0.98)	1.53 (1.44–1.65)	

\* Mean values and ranges of three experiments in plain 0.25 M sucrose solution

† 18.7 (18.2 to 19.6) m eq. of Mg per kilo of perfused liver tissue

‡ 551 (526 to 585) m eq. of Mg per 100 gm. of total nitrogen

the arginase-rich microsomes was higher than in the arginase-poor mitochondria or the arginase-rich nuclear fraction. Thus, there was no correlation between the distribution of magnesium and that of arginase in the cell fractions studied.

## DISCUSSION

The binding of arginase to nucleic acid-containing particles is in keeping with the observation of Moss (22) that the activity of arginase preparations is inhibited by adding yeast nucleic acid, and restored by increasing the manganese ion concentration. The ready release of particle-bound arginase by cations at or below their intracellular levels strongly suggests that the distribution pattern in electrolyte-free aqueous media is an artifact. It is probable that arginase, like the arginase-condensing enzyme (23), belongs to the soluble fraction of cytoplasm.

Recent electromicroscopic observations of Watson (24) indicate that the membrane of mammalian nuclei contains sufficiently large pores to permit passage of protein molecules. Anderson's (25) studies of isolated rat liver

nuclei have shown that penetration of the membrane by protein molecules is possible *in vitro*. There is thus evidence available in support of the view that the proteins of the soluble phases of nucleus and cytoplasm may be in diffusion equilibrium. This would explain the fact that arginase is found in both nucleus and cytoplasm of liver cells and that, with several of the isolation procedures employed by other investigators, the enzyme concentration in both cell components is identical.

Whether the affinity of arginase to nucleic acids has biological significance is obscure. Lang *et al.* (20) suggested that arginase is formed in the nucleus. The amount released into the cytoplasm could possibly be governed by the intracellular concentrations of free cations, but no evidence for this interpretation is as yet available. For the purification of arginase, however, its separation from the majority of soluble proteins by fractional centrifugation in electrolyte-free media and its subsequent release from the nucleic acid-containing particles by cations may be useful preliminary steps.

The danger of redistribution of enzymes through diffusion and adsorption upon disruption of the cell has been frequently mentioned, though known instances are rare. Our data indicate that the absence of cations in the homogenization medium may cause adsorption. However, for the effective separation of intracellular particulates by fractional centrifugation, electrolyte-free media are essential. Exposure of the separated particulate matter to salt solutions of intracellular composition seems to offer a simple check on the degree of association between enzyme and particle.

#### SUMMARY

After fractional centrifugation of rat liver homogenates in isotonic sucrose solution, 75 per cent of the arginase activity was recovered in the nuclear and microsomal fractions. Purification of the nuclei by density gradient centrifugation did not diminish their specific arginase activity. However, supplementation of the medium with 10 m eq per liter of divalent or 77 m eq per liter of monovalent cations resulted in practically complete elution of the enzyme. It is concluded that the localization pattern obtained in electrolyte-free sucrose solution is a preparative artifact caused by binding of arginase by nucleic acids. The enzyme appears to be a constituent of the soluble fraction of cytoplasm.

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# KYNURENINE AND 3-HYDROXYKYNURENINE AS PRECURSORS OF NIACIN IN THE RAT\*

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While kynurenine has been clearly implicated in the synthesis of niacin from tryptophan in *Neurospora crassa* (2, 3), there have been conflicting reports regarding its role as a niacin precursor in animals. It has been reported not to support growth (4) or to increase excretion of *N*<sup>1</sup>-methylnicotinamide (5) in rats. Other investigators found that kynurenine possesses niacin-replacing activity for growth (6) and for the stimulation of urinary *N*<sup>1</sup>-methylnicotinamide (7).

3-Hydroxykynurenine, which is involved in the formation of eye pigment by *Drosophila* (8), also appears to be an intermediate in the biosynthesis of niacin in *Neurospora* (9). The suggestion that this compound is a precursor of 3-hydroxyanthranilic acid has been supported by the demonstration that kynureninase catalyzes the removal of the side chain (10-12).

The ability, though limited, of kynurenine and 3-hydroxykynurenine to give rise to urinary niacin, quinolinate (13), and *N*<sup>1</sup>-methylnicotinamide (7) indicated that further studies of the capacity of these compounds to replace niacin for growth should be made. The results of experiments reported below indicate strongly that both compounds are effective in this regard, particularly when administered by the intraperitoneal route. Also presented are data obtained from studies *in vitro* which show that rat liver catalyzes the formation of quinolinic acid from hydroxykynurenine, but not from kynurenine.

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† Some of these data have been taken from a thesis submitted by R. E. Koski in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate College of the University of Illinois.

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## EXPERIMENTAL AND RESULTS

*Growth Experiments*—Weanling rats from the local colony, weighing 15 to 50 gm, were used to determine the niacin-replacing activity of kynurenine and hydroxykynurenine. For the first experiment, females were employed, males were used for the second and third experiments on growth.

A 9 per cent casein-sucrose diet supplemented with 0.2 per cent L-cystine and 0.1 per cent DL-threonine (14) was fed *ad libitum*. This diet results in a severe niacin-tryptophan deficiency in 1 to 2 weeks, and weanling rats not supplemented, rarely gain more than 20 to 30 gm in 5 weeks. The L-tryptophan, L-kynurenine sulfate, and 3-hydroxy-DL-kynurenine used in these studies were the materials previously described (13).

TABLE I

*Effect of L-Kynurenine and L-Tryptophan in Replacing Niacin for Rat Growth\**

Compound administered	Daily dosage	Average growth
	$\mu$ moles	gm per wk
Isotonic glucose		1.5
L-Kynurenine sulfate	25	10.0
“ “	50	9.7
L-Tryptophan	25	11.2

\* Animals were injected intraperitoneally with 25  $\mu$ moles per dose in 1 ml of isotonic glucose solution at pH 7.0. When 50  $\mu$ moles were administered, two injections were given, 6 hours apart.

In the first experiment, female rats which received the niacin-free diet were given L-kynurenine intraperitoneally each day for 3 weeks. Negative and positive control groups received isotonic glucose and 25  $\mu$ moles per day of L-tryptophan, respectively. The results (Table I) clearly demonstrate that L-kynurenine is approximately as active as additional tryptophan in supporting the growth of niacin-deficient animals under these conditions.

A second experiment was designed to compare the niacin-replacing activity of hydroxy-DL-kynurenine, DL-tryptophan, and niacin when given in the diet or by intraperitoneal injection. Both niacin and tryptophan supported growth at rates considered good for a 9 per cent protein diet, while hydroxykynurenine was less effective, especially when the compound was mixed in the diet (Table II). The group which received the 3-hydroxykynurenine by intraperitoneal injection at approximately the same level of intake grew better, but not as well as those animals receiving niacin or tryptophan. Although these results indicated that hydroxykynurenine can be converted to pyridine nucleotides in the rat, the results were not decisive as would be desirable.

Another experiment dealing with this point gave more definitely positive results. Male rats which had almost ceased to grow, owing to niacin deficiency, were caused to resume growth by injection of the proposed precursors of pyridine nucleotides. The results (Table III) clearly indi-

TABLE II  
*Effect of Hydroxy-DL-Kynurenine, Tryptophan, and Niacin on Growth Rate of Niacin-Deficient Rats\**

Supplement to deficient diet	Mode of administration	Growth, gm. per wk. for 5 wks
None		7.4
2.5 mg. % niacin	In diet	19.3
51 " % DL-tryptophan†	" "	25.8
56 " % hydroxy-DL-kynurenine†	" "	9.4
25 $\mu$ moles DL-tryptophan per day	Intraperitoneally	24.1
25 " hydroxy-DL-kynurenine	"	14.2

\* Four animals per group

† Ingestion of 10 gm. of diet per day being assumed, the dosage in these two groups would be 25  $\mu$ moles per day

TABLE III  
*Curative Effect of Hydroxy-DL-Kynurenine on Growth of Niacin-Deficient Rats\**

Compound injected, 25 $\mu$ moles per day†	Weight gain in 9 days	
	Mean	Range
None	2.2 gm	-2-+8 gm
L-Tryptophan	41.5	37-44
3-Hydroxyanthranilic acid	38.0	29-46
Hydroxy-DL-kynurenine	19.8	12-24

\* Four rats per group. All rats received the niacin-free, 9 per cent casein diet, supplemented with 0.1 per cent DL-threonine, until growth had ceased.

† Two intraperitoneal injections in isotonic saline 6 hours apart.

cated that hydroxykynurenine has niacin-replacing activity for the rat when given intraperitoneally, although it is less effective than tryptophan or 3-hydroxyanthranilic acid. No direct comparisons between kynurenine and hydroxykynurenine were made, but the DL form of the latter at 25  $\mu$ moles per day was approximately as effective for growth (Table II) as 25  $\mu$ moles of L-kynurenine (Table I). These studies on growth confirm the earlier report (6) that kynurenine is active and they support the excitation type of evidence (7, 13) that kynurenine and hydroxykynurenine are inter-



mediates or can be readily converted to intermediates in the transformation of tryptophan to niacin

*Studies in Vitro*—Attempts to form niacin from tryptophan in the presence of various types of surviving tissue have in general been unsuccessful. The formation of kynurenine from tryptophan in the presence of liver preparations occurs readily (15), but the presumed next step, the hydroxylation of kynurenine, has not been achieved. Products obtained in attempts to get this reaction to proceed *in vitro* include kynurenic acid, anthranilic acid, and a compound arising from anthranilic acid, which has been tentatively identified as a phenolic ester of 3-hydroxyanthranilic acid (16)

TABLE IV  
*Conversion of Kynurenine and Hydroxykynurenine to Niacin and Quinolinate by Liver and Kidney Preparations*

Tissue	Substrate	Increase in quinolate	Percent conversion of substrate to quinolate
Liver slices	None	0	0
" "	L-Kynurenine	12	1.6
" "	Hydroxy-DL-kynurenine	167	25
" "	3-Hydroxyanthranilate	612	92
" homogenate	L-Kynurenine	0	0
	Hydroxy-DL-kynurenine	169	25
	3-Hydroxyanthranilate	550	86
Kidney slices	L-Kynurenine	16	1.5
	Hydroxy-DL-kynurenine	20	2.1
Liver and kidney slices	L-Kynurenine	14	1.8
	Hydroxy-DL-kynurenine	113	15.3

In the experiments reported here, rat liver and kidney slices and homogenates were employed, alone and in combination, in efforts to convert kynurenine and hydroxykynurenine to 3-hydroxyanthranilate. Rat liver readily oxidizes the latter, almost quantitatively, to quinolinic acid; therefore, this system provides a very sensitive method for detecting the formation of 3-hydroxyanthranilate.

Tissues were prepared from adult male rats weighing approximately 300 gm. The slices and homogenates were incubated for 3 hours with substrates in Krebs-Ringer-phosphate buffer at pH 7.4 in an atmosphere of air, at 37°, with shaking. After being heated on a steam bath for 3 minutes the protein was removed and the filtrate chromatographed on Whatman No. 1 paper with the methanol-*n*-butanol-benzene-water (2:1:1:1) system. The niacin content before and after autoclaving with acetic acid (17) was determined microbiologically. The increase resulting from the acetic acid treatment is considered to be solely the result of decarboxylation of quin-

linic acid Chromatographic studies suggest that all of the nicotinic acid released by heating with glacial acetic acid arises from a substance with an  $R_F$  value corresponding to that of quinolinic acid

The results (Table IV) indicated that, while 25 per cent of the 3-hydroxy-DL-kynurenine was converted to quinolinate by either homogenates or slices of liver, an insignificant amount of kynurenine was converted to this product Kidney slices and homogenates failed to convert significant amounts of either substrate to quinolinate It seemed possible that hydroxylation of kynurenine could occur in kidney tissue which lacked the kynureninase to form 3-hydroxyanthranilate Consequently, both substrates were incubated with mixtures of liver and kidney slices The kynurenine was again not converted significantly, but hydroxykynurenine gave rise to quinolinate

The chromatographic results indicated that both anthranilic acid and kynurenic acid were being formed from kynurenine by liver slices, whereas kynurenic acid, but not anthranilic acid or its derivatives, was produced by kidney No evidence for the formation of hydroxykynurenine or xanthurenic acid or their derivatives was obtained with kidney, but xanthurenic acid did appear when the substrate was hydroxykynurenine The chromatographic data obtained are consistent with the view that under these conditions kynurenine is not hydroxylated by liver or kidney or both, but that hydroxykynurenine is converted to hydroxyanthranilate by liver, but not by kidney

#### DISCUSSION

Kynurenine and hydroxykynurenine are about equally effective as precursors of urinary quinolinate *in vivo* These compounds are metabolized in such a manner that xanthurenic acid is a more important excretion product than quinolinic acid, whereas tryptophan gives more quinolinic acid in normal rats (13) Thus, it is evident that, if kynurenine and hydroxykynurenine are direct intermediates in the formation of quinolinate, the provision of these compounds *per se* leads to a distinctly different metabolism quantitatively than if they arise from tryptophan within the animal The results do not exclude the possibility that derivatives of kynurenine and hydroxykynurenine are the true intermediates The biological activity of these compounds could result from limited conversion to the more active derivatives, as has been suggested (18), but such derivatives have not yet been identified

The failure of the tissues tested to result of choosing the tissue kynureninase and substrate for this kynurenine in reported by

DL-kyn... may be the... with... the true... tion...  
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slight conversion of kynurenine to quinolinate (1 to 2 per cent) noted in these studies may be the result of slight non-enzymatic hydroxylation. In the hydroxylation is as slow in the rat as the excretion studies indicate (13), this non-specific hydroxylation might account for all of the xanthurenic acid and quinolinic acid formed from kynurenine. On the other hand, the major part of tryptophan given to vitamin B<sub>6</sub>-deficient rats is converted to xanthurenic acid, presumably via hydroxylation of kynurenine or a closely related compound. It is doubtful whether non-specific hydroxylation could account for this rapid transformation.<sup>1</sup>

#### SUMMARY

1 L-Kynurenine sulfate and 3-hydroxy-DL-kynurenine support the growth of niacin-deficient rats somewhat less effectively than tryptophan. The latter compound is more effective when injected than when incorporated into the diet.

2 Rat liver slices and homogenates catalyze the conversion of hydroxykynurenine to quinolinate, but do not hydroxylate kynurenine appreciably. Kidney slices were inactive in converting kynurenine or 3-hydroxykynurenine to quinolinate.

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<sup>1</sup> A recent communication by De Castro *et al.* (20) reports the hydroxylation of kynurenine by washed mitochondria from rat and cat livers, where reduced nicotinamide nucleotide is provided.

# PAPER CHROMATOGRAPHY OF PHOSPHOLIPIDES\*

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Previous studies (1-7) on the paper chromatography of phospholipides have in most cases been limited to a few individual pure lipides, very little having been reported on the chromatography of total phospholipide extracts of tissues (5). Furthermore, there have been no publications dealing with factors which influence the paper chromatographic behavior of the phospholipides. Studies along these lines are presented in this communication.

An investigation was first made of the behavior of pure phospholipides of known structure. This work gave important information on how factors such as the mode of application of the lipides to the paper, concentration, degree of unsaturation, chain length, ionic groups of the lipides, and solvent polarity influence the mobilities of these compounds. Individual solvents were studied first and then mixtures of the solvents were devised. Ionic solvents consisting of mixtures of neutral organic solvents with organic acids or bases, or with a mixture of an acid and a base, proved to be the most promising.

These solvent systems gave good results on lipide extracts of a variety of rat tissues. It is felt, therefore, that the experimental data presented in this paper should be useful to both those investigating the metabolism of the phospholipides and those interested in studying the phospholipide composition of tissues on a micro scale.

## *Techniques and Reagents*

*Pure Phospholipides*—Synthetic lecithins, cephalins,<sup>1</sup> glycollecithin,<sup>1</sup> and phosphatidic acid were obtained through the courtesy of Dr. E. Baer.

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<sup>1</sup> Cephalin designates diacylglycerophosphorylethanolamine (or phosphatidylethanolamine), glycollecithin designates the "lecithin" analogue containing ethylene glycol in place of glycerol, hydrolecithin designates the naturally occurring dipalmitylecithin.

at the University of Toronto, and dipalmitoleyllecithin was a gift of D. D. Hanahan of the University of Washington. Monopalmitoleyllecithin (lys-lecithin) was prepared as described by Hanahan (8), ceramide by the method of Tropp and Wiedersheim (9), and sphingomyelin and hydroxy-lecithin<sup>1</sup> were obtained by the procedure of Thannhauser and Boncompagni (10). Cerebroside was prepared from beef brain. The purity of the compounds was checked by elementary analysis and by infrared spectroscopy (11).

*Solvents*—The following solvents were used: baker's analyzed acetic acid, chloroform, and isoamyl alcohol, Mallinckrodt analytical grade absolute methanol, and Eastman Kodak 2-octanol (white label) and lutid (lot No. T 1809). Mixed solvents were prepared by volume.

*Other Reagents*—The fluorescent dyes, rhodamine B and G and fast acid violet GRF, were obtained from the Biological Stain Commission and were samples of dyes from the National Aniline Division, Allied Chemical and Dye Corporation, Buffalo, New York.

*Chromatographic Chambers*—The papers were run by the ascending technique in chambers which were previously equilibrated with the solvent for about 8 hours, with the exception of those containing octanol-acetic acid solvent, which were equilibrated for about 2 hours. Cylindrical glass chambers were used which have an inside diameter of 6, 10, or 12 inches and a height of 18 inches. These were lined with filter paper which was wet with the developing solvent. Equilibration of the chromatographic paper in the chamber before beginning the run was found to be unnecessary since no change in the  $R_F$  values of the phospholipides was observed.

*Filter Paper*—Whatman No. 1 paper was washed extensively by the descending technique with 2 *N* aqueous acetic acid. In some cases the paper was further washed with water and then with methanol. After thorough drying by air, the papers were heated at 105° for 15 minutes and then stored in a closed drawer. Our experience with phospholipides confirms the work with carboxylic acids and water-soluble phosphate esters in which others have shown that spot distortion can be minimized by acid washing (12, 13).

*Detection Methods*—Before the detection methods were used, the chromatograms were dried in air at room temperature from 8 to 24 hours, washed in distilled water for 10 minutes, and again dried in air. Thus not only removed excess solvent which, when present, decreased the sensitivity of the test reagents, but also eliminated certain water-soluble substances such as orthophosphate, phosphate esters, nucleotides, urea, and amino acids which might interfere with some of the detection tests in the case of a lipid extract of a tissue. The washing procedure did not cause a loss of lipid from the paper.

Staining reaction with rhodamines B or G, or with fast acid violet GRF

was used as a general test for all the lipides. The chromatograms were dipped into a 0.001 per cent aqueous solution of the dye, allowed to stand about 10 minutes, and then washed for a few minutes with water to remove the excess dye. The lipides were stained by these dyes and exhibited either yellow, pink, orange, or blue fluorescence under ultraviolet light.<sup>2</sup> There was a considerable variation in the sensitivity of the dye with change in solvent system and with different lipides. For the detection of phosphate esters, the Hanes and Isherwood reagent (13) was sprayed on the chromatograms, which were then allowed to stand for 30 minutes to 1 hour at room temperature and placed in an oven at 85° for 8 to 15 minutes. After removal from the oven, the paper was exposed to daylight until the blue phosphate areas were visible. Lipides which contain a free amino group were detected by spraying the chromatograms with a 0.3 per cent solution of ninhydrin in *n*-butanol saturated with water and containing 5 per cent lutidine. After standing in subdued light at room temperature, the blue colors developed. Unsaturated lipides were detected by dipping the chromatograms into a 1 per cent aqueous solution of  $\text{KMnO}_4$  for 1 to 2 minutes and washing out the excess permanganate with running water. The lipides appeared as brown spots. Choline-containing lipides were identified by the method of Levine and Chargaff (14). Chromatograms must be washed free of lutidine for a satisfactory test. The limit of sensitivity of the dye and ninhydrin tests was about 10  $\gamma$ , whereas with the other tests it was about 50  $\gamma$ .

*Application of Lipides to Paper*—The method of application of the lipides to the paper was found to be an important factor in determining the over-all chromatographic picture. Each phospholipide was applied on a line  $2\frac{1}{2}$  inches from the lower edge of the paper at a concentration varying from 10 to 100  $\gamma$  in a volume of 20  $\mu\text{l}$ . Two solvents were found to be satisfactory for the application of the phospholipides. The first, isoamyl alcohol-benzene (1:1), gave uniform distribution of the lipide over the spot area, was stable, dissolved all the lipides except purified acetal phospholipide, and gave rise to satisfactory spots when the chromatograms were developed. The second solvent, lutidine-acetic acid (3:1), gave similar results and in addition sometimes yielded better spots. However, the  $R_F$  values obtained after application in the latter solvent were lower than when isoamyl alcohol-benzene (1:1) was used, and the lutidine-acetic acid mixture became discolored on standing for a few days, even in the cold. The isoamyl alcohol-benzene mixture was used in almost all of the experiments. Acetal phospholipide was applied in hot lutidine-acetic acid or in wet chloroform.

<sup>2</sup> A. G. W. Gates and Company, Lamp, 366  $\text{m}\mu$  wave length was used as the source of ultraviolet light.

## Results

*Movement of Phospholipides in Individual Solvents*—The first step in the development of solvent systems was to determine the capability of various individual solvents to move the phospholipides on paper. The results

TABLE I  
*Relationship of Solvent Polarity and Phospholipide Solubility  
to Chromatographic Behavior of Phospholipides\**

	Individual solvent	Relative mobility of lecithin, cephalin, and sphingomyelin	Relative solubility† of lecithin, cephalin, and sphingomyelin in solvent
Polar	Alcohols (methanol)	Near solvent front	Moderately soluble
	Carboxylic acids (acetic acid)	To solvent front	Very soluble
	Primary amines (propyl amine)	Slight movement	Slightly soluble
Slightly polar	Alkyl halides (chloroform)	" "	Very soluble
	Ketones (acetone)	" "	" slightly soluble
	Aldehydes (benzaldehyde)	" "	" " "
	Esters (ethyl acetate)	" "	" " "
	Ethers (n-butyl ether)	" "	" " "
	Tertiary amines (lutidine)	" "	" " "
Non polar	Hydrocarbons (benzene)	Very slight	" soluble
	Alkyl halides (carbon tetrachloride)	" "	" "

\* The phospholipides were applied in isoamyl alcohol-benzene (1:1) at a concentration of 30  $\gamma$  per 10  $\mu$ l.

† Other solvents tested which gave similar results to the solvents given above are polar solvents, ethanol, propanol, *n*-, *sec*-, and *tert*-butanol, amyl and isoamyl alcohol, hexanol, 2-octanol, decanol, formic, propionic, butyric, and isobutyric acids, methyl, ethyl, and butyl amines, slightly polar solvents, diethyl ketone, methyl isopropyl ketone, disobutyl ketone, cyclohexanone, propionaldehyde, dimethyl ether, dioxane, isopropyl ether, diethyl ether, pyridine, collidine, quinoline, and non-polar solvents, hexane, cyclohexane.

‡ Relative solubility at room temperature

(Table I) show that solubility of the lipide in the solvent is not the sole factor governing the mobility of the lipides, another appears to be the polarity of the developing solvent. If solvents with somewhat similar properties with respect to the solubility of the lipides are arranged in order of polarity (e.g. carbon tetrachloride, chloroform, methanol), the mobility is found to increase with increase in polarity. These results indicate that the polarity

pholipide molecules are bound to the paper or interact with each other in such a way that a solvent must be sufficiently polar to break these binding forces in order to cause them to migrate

*Movement of Phospholipides in Solvent Mixtures*—To obtain mobility of the phospholipides, at least one of the components of the mixture must be polar (Table I). Thus, when the relatively non-polar lutidine, which did not move the phospholipide, was mixed with methanol satisfactory movement of the lipides occurred. Although it may appear that a mixture of two polar solvents would not be useful, since movement of all the lipides to the front might occur, it was found that the  $R_F$  values of the phospholipides in such mixtures were less than the high values observed with either polar solvent alone, and that satisfactory solvent systems were obtained if the concentration of the more polar constituent was kept relatively small. An example of this type of solvent is octanol-acetic acid (99:1) (Table II). Other solvents devised on this principle and containing mixtures of lutidine and acetic acid, together with an excess of chloroform or an alcohol, were developed (Table II). Attempts to improve these systems by the addition of aldehydes, ketones, ethers, esters, or hydrocarbons were unsuccessful since these resulted in increased streaking or spot elongation. The composition of the satisfactory solvent systems (Table II) can be varied within fairly wide limits in most cases to allow the use of the same general type of system for different lipide mixtures. Since the movement of the lipides is influenced not only by the concentration of the various lipides present but also by the composition of the lipide mixture, it seems reasonable to assume that the solvent systems might have to be adjusted in order to obtain the best chromatograms with the particular lipide mixture under investigation. Thus, the composition of the octanol-acetic acid system can be varied from 99:1 to 99:4, that of methanol-lutidine-acetic acid from 8:16:1 to 4:16:1, and that of chloroform-lutidine-acetic acid from 4:4:1 to 8:4:1.

From the  $R_F$  values (Table II) certain general conclusions could be drawn concerning the movement of phospholipides on paper. Anionic or neutral lipides moved to or near the solvent front in all the systems. The purified acetal phospholipide, which is believed to differ structurally from the native plasmalogen (15, 16), showed essentially no movement, and cephalins had a tendency to streak. The behavior of these latter lipides is undoubtedly related in part to their solubility which is much lower than that of the other phospholipides in the solvents used. There was a marked increase in the mobility of the cephalins with decrease in chain length. In contrast, lecithins usually exhibited a slight decrease in mobility with decrease in chain length. The greatest change in mobility owing to unsaturation of the fatty acid chains of lecithin was noted in Solvent System C. Although no synthetic unsaturated cephalin was studied, results with tissue lipides (Fig. 1)



showed that unsaturated naturally occurring cephalins moved similarly to the shorter chain synthetic dimyristylcephalin. Since naturally occurring cephalins contain fatty acids predominantly  $C_{18}$  and higher, unsaturation in these compounds increases their mobility.

TABLE II  
*R<sub>F</sub> Values of Pure Phospholipides\**

The lipides were applied in 20  $\mu$ l of isoamyl alcohol-benzene (1:1) except for acetal phospholipide, which was applied in hot lutidine-acetic acid (3:1). 100  $\gamma$  of cholesterol, cholesterol palmitate, tristearin, oleic acid, stearic acid, monopalmitin, phosphatidic acid, or ceramide were used, 30  $\gamma$  of each of the other phospholipides were employed.

Lipide	Solvent A†	Solvent B†	Solvent C†	Solvent D†
Dimyristoylleceithin	0.48	0.31	0.40	0.21
Dipalmitoylleceithin	0.50	0.34	0.40	0.27
Distearoylleceithin	0.50	0.30	0.40	0.27
Dipalmitoleylleceithin	0.53	0.37	0.53	0.29
Monopalmitoleylleceithin	0.14	0.29	0.15	0.27
Dimyristoylcephalin	0.46‡	0.41‡	0.34	0.10§
Distearoylcephalin	0.05§	0.12§	0.15§	0.03§
Acetal phosphatide	0.00	0.00-0.03	0.00	0.00
Sphingomyelin	0.38	0.33	0.40	0.26
Phosphatidic acid	0.95	0.95	0.95	0.95
Stearoylglycolleceithin	0.39	0.13	0.30	0.24
Ceramide	0.93	0.93	0.93	0.93
Cerebroside	0.85	0.90		
Other Lipides	>0.90	>0.90	>0.90	>0.90

\* The  $R_F$  values showed some variation owing to temperature changes and solvent changes on standing for several days.

† The solvent systems had the following composition (v/v): Solvent A, chloroform-lutidine-acetic acid (4:4:1), Solvent B, lutidine-methanol-acetic acid (16:4:1), Solvent C, 2-octanol-lutidine-acetic acid (90:5:5), Solvent D, 2-octanol-acetic acid (99:1).

‡ Exhibited tailing.

§ Exhibited streaking forward.

|| Other lipides include palmitic, stearic, and oleic acids, mono-, di-, and tri-glycerides, cholesterol, and cholesterol palmitate.

**Separation of Mixtures**—The usefulness of the solvent systems was tested by application to model lipid mixtures. Under the conditions given in Table II, the anionic or neutral lipides which ran near the front could be readily separated in all the solvents from all the phospholipides except phosphatidic acid. Thus, this method could be applied not only to phospholipide but to total lipid extracts. Mixtures of lysolecithin, lecithin, and oleic acid were separated in chloroform-lutidine-acetic acid. Acetal phospholipide

pholipide, lecithin, and phosphatidic acid were separated from each other in methanol-lutidine-acetic acid

At the concentrations given in Table II, it was difficult to achieve clear-cut separations of lecithin and cephalin or sphingomyelin because the spots tended to fuse together. However, when the concentration of these lipides was reduced to 10 to 15  $\gamma$  per 20  $\mu$ l and the spots were applied in lutidine-acetic acid, clean-cut separations of the pairs sphingomyelin and dimyristylcephalin or sphingomyelin and distearylcephalin were obtained with the chloroform-lutidine-acetic acid solvent, whereas the mixtures distearyl-

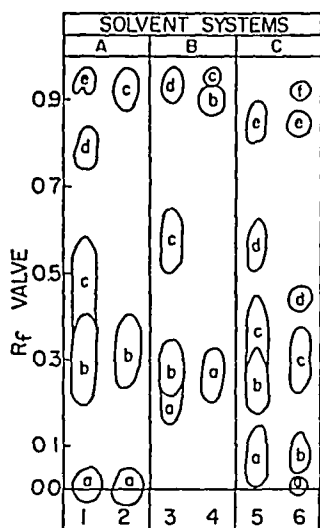


Fig 1 Chromatograms of phospholipides from rat liver. The results are given as scale drawings of autoradiograms, which were developed on "no screen" x-ray film. The tests given by each spot are listed in Table III. In scale drawings Nos 1, 3, and 5, the phospholipides were applied at a concentration of 60  $\gamma$  per 20  $\mu$ l, with Nos 2, 4, and 6 the acetylated phospholipides were applied at a concentration of 160  $\gamma$  per 20  $\mu$ l. The composition of the chromatographic solvents is given in Table II.

lecithin and distearylcephalin or distearyllecithin and dimyristylcephalin were resolved in the octanol-acetic acid solvent. Similarly, separation of distearyllecithin and distearylcephalin was achieved in octanol-lutidine-acetic acid. The model mixtures just discussed represent only a few examples of a larger number of mixtures which should be capable of separation. The acylation technique reported elsewhere (17) also gave clean-cut separations of cephalin and acetal phospholipide from lecithin and sphingomyelin, but allowed the use of higher concentrations of lipides.

*Two-Dimensional Chromatography and Paper Modification*—Attempts to increase the range of separations by the use of two-dimensional chromatography were unsuccessful. When the second developing system was Solvent A or C, no movement of the phospholipides was observed, when the second

system was Solvent B, the spots obtained were elongated and considerable streaking resulted

Investigations of paper oxidized with periodic acid, and of filter paper impregnated with mineral oil, rubber, silicone, formamide, or silicic acid showed that only the silicic acid paper was successful. The results of studies with papers impregnated with silicic acid have been published elsewhere.<sup>3</sup> Recently Lea, Rhodes, and Stoll (18) and Dieckert and Reiser (19) have successfully used silicic acid impregnation for phospholipid chromatography. Experiments now in progress show that the solvents in Table II are particularly suitable for circular chromatography.

*Application to Rat Tissues*—The chromatographic systems were applied to total lipid extracts of heart and liver of white rats which were injected subcutaneously with  $P^{32}$  inorganic phosphate. In the diagrams of the autoradiograms of the liver phospholipides (Fig. 1), identification of the lipid spots on the chromatograms was made by chemical tests, comparison of their  $R_F$  values with known lipids, and their behavior after acylation (17) and selective alkaline hydrolysis. These data are summarized in Table III. The major phospholipides of both tissues were found to be lecithin and cephalin, which occurred in highest amount and were heavily labeled. The minor components corresponded to sphingomyelin, phosphatidyl serine, acetal phosphatide, and possibly inositol phosphatide. The native acetal phosphatide moved much faster than the corresponding reference compound but could be identified by its increase in mobility upon acylation. Mild alkaline hydrolysis of heart lipids prior to chromatography gave rise to a new ninhydrin-reacting component which, like the reference acetal phosphatide, showed essentially no mobility. In view of the fact that the reference compound was purified by alkaline treatment, it is clear that the structure of this latter lipid has been altered during the course of purification.

The failure to find a spot having an  $R_F$  value greater than 0.9 in the octanol-lutidine-acetic acid solvent, even when a large excess of total lipid was used, demonstrates the absence of diacylphosphatidic acid in both liver and heart. Since this lipid is assumed to be an intermediate in the biosynthesis of lecithin by rat liver systems (20, 21), the failure to find phosphatidic acid casts doubt on its role in this process. Further studies with rat brain also confirmed the absence of phosphatidic acid. Dawson (22) has stated that a compound similar to phosphatidic acid is readily labeled with  $P^{32}$  *in vitro*. This apparent discrepancy is undoubtedly related to different metabolic pathways between *in vivo* and *in vitro* systems.

The results obtained with rat liver and heart also show the presence of unidentified phospholipids which occur in small concentration and behave like none of the known lipids studied. Since some of these show an *in*

<sup>3</sup> Accepted for publication (Witter, R., *Biochim. et biophys. acta*, **21**, 168 (1956))

TABLE III  
Spot Tests for Lipides in Fig 1

All spots gave permanganate and rhodamine B or G dye tests. Radioactive lipides were detected by the darkening of the film used for autoradiograms. The degree of darkening of the film is designated as follows: +s = strong positive test, +m = moderately strong positive test, +w = weak positive test, +vw = very weak positive test. A negative test is indicated by —

Before acetylation					After acetylation				
Spot No	pn	Nin hy-drin	Cho line	Lipide components*	Spot No	pn	Nin hy-drin	Cho line	Lipide components*
1a	+m	—	—	Unidentified PL	2a	+m	—	—	Unidentified PL
1b	+s	+	+	Lecithin and small amounts of sphingomyelin and cephalin	2b	+s	—	+	Lecithin and small amounts of sphingomyelin
1c	+s	+	—	Cephalin and small amount acetal PL	2c	+s	—	—	Acetylcephalin, acetylacetal PL, acetylserine PL and other unidentified PL
1d	+m	—	—	Serine PL†					
1e	+w	—	—	Unidentified PL					
3a	+s	—	+	Lecithin and small amount sphingomyelin	4a	+s	—	+	Lecithin and small amounts of sphingomyelin
3b	+s	+	—	Cephalin and small amount acetal PL	4b	+s	—	+	Acetylcephalin, acetylserine PL, and unidentified PL
3c	+m	—	—	Serine PL†	4c	+w	—	—	Acetylacetal PL and unidentified PL
3d	+w	—	+	Unidentified PL					
5a	+w	+	—	Acetal PL and other unidentified PL	6a	+m	—	—	Unidentified PL
5b	+s	+	+†	Cephalin	6b	+m	—	+†	" "
5c	+s	—	+	Lecithin and small amounts sphingomyelin	6c	+s	—	+	Lecithin and small amounts sphingomyelin
5d	+w	—	—	Serine PL†	6d	+s	—	+	Unidentified PL
5e	+vw	—	—	Unidentified PL	6e	+s	—	—	Acetylcephalin, acetylserine PL and unidentified PL
					6f	+w	—	—	Unidentified PL or acetylacetal PL

\* PL = phospholipide

† Identification of phosphatidyl serine is tentative. This spot failed to give a positive test with ninhydrin because it occurred in too low a concentration.

‡ Only the upper part of the spot gave test

creased mobility after acylation, they are believed to represent new types of amino-containing phospholipides. The present studies indicate that the phospholipide composition of rat tissues is more complex than formerly believed.

#### DISCUSSION

From the experimental data it is possible to offer an explanation on theoretical grounds concerning the chromatographic behavior of phospholipides on paper. Such a hypothesis is useful not only for the explanation of the results presented in this paper, but also as an aid for the design of new solvent systems and for the prediction of the chromatographic behavior of the phospholipides.

The hypothesis is based on the observation that a mixture of an excess of a relatively less polar solvent with a small amount of a more polar solvent caused the phospholipides to move with intermediate  $R_F$  values (0.3 to 0.6), whereas either solvent alone caused movement to or near the solvent front. This strongly suggests that the homogeneous solvent system separated on the paper into two phases. An example of this type of system is Solvent D (Table II) in which octanol and acetic acid are mixed in a ratio of 99:1. The more polar solvent (acetic acid), present in low concentration, is believed to be more firmly bound to the paper and thus to constitute the stationary phase, whereas the less polar constituent (octanol) comprises the mobile phase. The polar phase bound to the paper apparently exerts a retarding effect on the movement of the phospholipides, since they are more soluble in this phase. Increase in the concentration of the more polar component (acetic acid), which constitutes the stationary phase, results in greater migration of the phospholipides until the point is reached (for example, octanol-acetic acid (95:5)), where the phospholipides move near the solvent front as they did when the two solvents were used separately. Apparently this occurs because, as the concentration of the more firmly bound solvent (acetic acid) is raised, the capacity of the paper to bind it is exceeded, with the result that more and more of this component "spill over" into the mobile phase (octanol) with subsequent increase in the mobility of the phospholipides.

The major constituents of the stationary and mobile phases of the other chromatographic solvents given in Table II can also be deduced if the assumption is made that the forces or factors which bind the individual components of these solvents to the paper decrease in this order: acetic acid, alcohol, lutidine, and chloroform. Thus the solvents devised are of the following types: acid-stationary phase (octanol-acetic acid and octanol-lutidine-acetic acid), base-stationary phase (chloroform-lutidine-acetic acid), and alcohol-stationary phase (methanol-lutidine-acetic acid). In

solvents containing both lutidine and acetic acid, the lutidinium acetate formed is believed to be distributed between both the mobile and the stationary phases. In Solvents A and C, this salt may play a significant role in the stationary phase. The phospholipides probably exist as double salts of lutidinium acetate in solvents containing both lutidine and acetic acid. On the other hand, in the octanol-acetic acid solvent these lipides probably move as the acetate salt.

The data in Table I indicate quite clearly that the phospholipides are firmly bound to the paper and that polar solvents are required to disrupt these forces. Since filter paper possesses a negative charge, electrostatic forces in part contribute to this binding. A similar situation exists in the paper electrophoresis of proteins (23, 24). In addition, the polyhydroxy nature of the filter paper permits secondary forces such as hydrogen bonding to exert an effect in this regard. In support of these assumptions are the experimental observations that phospholipides having a positive charge, *i.e.* zwitter ions, had much lower mobilities than neutral or anionic lipides. Furthermore, the removal of the positive charge on the amino-containing phospholipides by acylation produced a large increase in their mobilities.

Phospholipides with a free hydroxyl group moved slower than corresponding lipides not having this group. The effect of a free hydroxyl group, however, in some instances was difficult to assess because of the influence of other factors. Although lysolecithin moved more slowly than lecithin, part of this lowering in mobility may be attributed to the increase in polarity of lysolecithin brought about by a reduction in its hydrocarbon nature owing to the fact that it contains one fatty acid chain, whereas lecithin contains two such chains. In this regard, lysolecithin behaved similarly to glycollecithin. The decrease in the hydrocarbon moiety of these latter lipides makes the contribution of the polar phosphorylcholine group to their mobility much more pronounced. Indeed, the increase in polarity is apparently large enough to offset their increased solubility in the solvents.

The slower mobility of sphingomyelin with respect to lecithin in Solvent A of Table II may be attributed to its having both an NH and an OH group, but also may be in part due to its lower solubility. It seems reasonable that phospholipides having an OH or NH group could possess lower mobilities than corresponding lipides which lack these groups, since hydrogen bonding with the filter paper can occur. Moreover, this effect should be more pronounced in less polar solvents. The experimental observations support this belief since lysolecithin and sphingomyelin displayed a more pronounced decrease in mobility with respect to lecithin only in the less polar system (Solvent A).

Another illustration of the interplay of various factors in determining

lipide mobility is shown by the difference in behavior of homologous lecithins as compared to homologous cephalins. With the lecithins which contain a strongly positive quaternary nitrogen atom, a decrease in chain length would make the effect of the positive charge more predominant and thus explain in part the decrease in  $R_F$  values with decrease in chain length of these compounds. On the other hand, the cephalins contain a weaker basic group and consequently a weaker positive charge on the nitrogen atom, therefore, a decrease in chain length in the cephalins would not be expected to produce so great an effect on their mobility. Indeed, in contrast to the lecithins, the cephalins increased in mobility with decreasing chain length. Part of the difference in the movement of individual cephalins may be due to the fact that distearylcephalin is much more insoluble than dimyristylcephalin.

#### SUMMARY

1 A study of a variety of individual solvents and solvent mixtures showed that polar or ionic solvents were most suitable for the chromatography of phospholipides on non-impregnated filter paper. Mixtures of lutidine and acetic acid with alcohols or with chloroform were found to give satisfactory results.

2 Many useful lipide separations were accomplished, such as lecithin from sphingomyelin and cephalin, cephalin from sphingomyelin, lysolecithin from lecithin, acetal phospholipide or phosphatidic acid from all other phospholipides, and phospholipides (except phosphatidic acid) from fatty acids, cholesterol, cholesterol palmitate, ceramide, and mono-, di-, and triglycerides. The separation of lecithin from either sphingomyelin or cephalin was accomplished only when small amounts of lipides were used. Larger amounts led to spot elongation and only partial separation.

3 Factors which influence the mobility of the phospholipides on paper were found to be solvent polarity, temperature, mode of application of the lipides to the paper, and lipide solubility, concentration, and structure.

4 These solvents were applied to  $P^{32}$ -labeled phospholipides of rat tissues. The presence of diacyl-phosphatidic acid could not be demonstrated, but several unidentified components were detected in these tissues and are believed to represent new phospholipides.

5 An explanation is presented of the chromatographic behavior of the phospholipides on filter paper.

*Addendum*—After the manuscript was completed we received a sample of brain diphosphoinositide through the courtesy of Mrs. Catherine F. McPherson of the Department of Biochemistry of the University of Western Ontario. This material, applied in hot isoamyl alcohol-benzene (1:1) or in wet chloroform, showed essentially no movement in any of the solvent systems. Most of the lipide remained at the origin, but a slight streaking forward to about  $R_F$  0.2 was observed.

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# CHARACTERIZATION AND PROPERTIES OF 2-KETO-3-DEOXY-D-ARABONIC ACID\*

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A new pathway for the oxidation of D-arabinose has been found recently in *Pseudomonas saccharophila* (1). The following sequence of events has been deduced from experiments with cell-free preparations. D-arabinose is oxidized in a diphosphopyridine nucleotide (DPN)-linked reaction to D-arabonic acid. The first product is probably D-arabono- $\gamma$ -lactone, which is hydrolyzed rapidly by an enzyme to yield the free acid. D-Arabonic acid is then converted to a compound which appears to be 2-keto-3-deoxy-D-arabonic acid. This intermediate is oxidized with 1 mole of DPN to yield equimolar amounts of pyruvic and glycolic acids. Tracer experiments have shown that the carboxyl group of pyruvic acid is derived from C<sub>1</sub> of arabonic acid and the carbinol group of glycolic acid from C<sub>5</sub>. The accumulation of considerable amounts of glycolic acid in cultures grown with D-arabinose as substrate indicates that the above mechanism operates *in vivo*.

The following experiments were conducted with a view to establishing the structure of the relatively unstable intermediate, 2-keto-3-deoxy-D-arabonic acid.

## Methods

The D-arabinose strain of *P. saccharophila* was cultivated in the medium previously described (2), but which contains 0.25 per cent D-arabinose as the source of carbon in place of fructose. The cultures were incubated at 30° with constant shaking for 16 to 24 hours, and the cells were harvested by centrifugation, washed twice with water, and kept in the frozen state. Extracts were obtained by grinding the cells with levigated alumina (3) in a cold mortar, 4 parts of buffer (in terms of the wet weight of the cells) were added, and the suspension was centrifuged in the cold at 22,000  $\times g$  for 30 minutes. Extracts have also been obtained by sonic disruption in a 9 kc Raytheon apparatus for 20 minutes in the cold. DPN reduction was stud-

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ied at 340  $m\mu$  in the Beckman model DU quartz spectrophotometer. Silica cells of 1 cm light path were used in all the determinations. Manometric experiments were performed with the conventional Warburg technique at 30°. The washed particulate fraction of *Azotobacter vinelandii* was used as a source of DPNH oxidase by the procedure of D. A. J. Marr (unpublished).

D-Arabonic acid was prepared from D-arabinose by the procedure of Moore and Link (4). Arabono- $\gamma$ -lactone was estimated by hydroxamate acid formation (5). 2-Keto-3-deoxy-D-arabonic acid was measured as the quinoxaline derivative by the method of Lanning and Cohen (6), or as the semicarbazone by the procedure of MacGee and Doudoroff (7). These methods were standardized against estimation of the keto acid by decarboxylation with ceric sulfate (8). Pyruvic acid was measured as the 2,4-dinitrophenylhydrazone, by the method of Friedemann and Haugen (9). Glycolic acid was estimated according to the procedure of Dagley and Rodgers (10) and formaldehyde by the method of MacFadyen (11).

For the paper chromatographic procedures, the following solvents have been used. Solvent I, methyl Cellosolve-water-concentrated ammonia (80:15:5), II, *n*-propanol-formic acid-water (6:3:1), III, ether-benzene-formic acid-water (70:30:14:10), IV, *n*-butanol-acetic acid-water (4:1:5, upper layer), V, *n*-butanol saturated with a 3 per cent solution of concentrated ammonia in water, VI, *tert*-butanol-methylethylketone-water-diethylamine (40:40:20:4), VII, *n*-propanol-water-diethylamine (85:15:3), VIII, methanol-water-pyridine (80:20:4), IX, methanol-water-pyridine (60:20:20), and X, collidine-water (125:44) saturated with 0.067 M boric acid in 0.067 M KCl, pH 9.0. The paper was sprayed before use with the same borate buffer.

To detect 2-keto-3-deoxy-D-arabonic acid, the chromatograms were sprayed with the alkaline silver nitrate reagent of Trevelyan *et al* (12), with the *o*-phenylenediamine reagent (6), the semicarbazide spray (13), or acid base indicators. The amino acids could be located with a 0.1 per cent ninhydrin in 95 per cent ethanol containing 0.5 per cent collidine. Lactones were detected on paper by a slight modification of the method of Abdel Akher and Smith (14) and for hydroxamates, a saturated solution of ferric chloride in *n*-butanol, saturated with water, was used as a spray (15). 2,4-Dinitrophenylhydrazones of  $\alpha$ -keto acids were prepared for paper chromatography by the method of Cavallini *et al* (16). Other methods will be mentioned in the text.

#### EXPERIMENTAL

*Preparation of Crude Calcium Salt of 2-Keto-3-deoxy-D-arabonic Acid*—When potassium arabonate was incubated at pH 6.8 with crude enzyme

preparations, its conversion to an active intermediate could be traced by any of the following means (1) the disappearance of arabonate, as measured by the decrease in the amount of substrate which could be lactonized with HCl and converted to hydroxamic acid, (2) the production of a compound which gave the characteristic quinoxaline spectrum after treatment with *o*-phenylenediamine and was decarboxylated with ceric sulfate, and (3) the appearance of a substance which caused an immediate reduction of DPN in the presence of the crude enzyme at pH 9.0. By these methods it could be shown that most of the arabonate initially added could be converted by the enzyme.

Although the keto acid could be precipitated as a calcium salt and was chromatographically separable from D-arabonic acid, all attempts to obtain it in pure form have so far been unsuccessful. This is largely attributable to the relative instability of the substance, since great losses of the compound were sustained upon treatment of the reaction mixtures with acid or with ion exchange resins as well as during the chromatography of the salts. In fact, the compound disappeared completely on some chromatograms, and the best precipitates obtained usually had a lower content of the keto acid than was expected from the observed conversion of arabonate in the enzyme preparations.

The following procedure was used to obtain a crude calcium salt of approximately 67 per cent purity in 26.2 per cent yield from arabonate. 33 ml of enzyme solution prepared from 1 part of cells ground with 4 parts of 0.01 M phosphate buffer, pH 6.8, were incubated with 3 ml of a solution of potassium D-arabonate containing 3.94 mmoles, for 12 hours at 30°. During this time, 3.02 mmoles of arabonate were converted to  $\alpha$ -keto acid, as determined with ceric sulfate decarboxylation. The mixture was chilled and treated with Dowex 50 in the H form to remove cations. The protein precipitate was removed by filtration. The solution was carefully adjusted to pH 7.5 by the slow addition of a suspension of  $\text{Ca}(\text{OH})_2$ , and 1 volume of methanol was added. The precipitate was removed by centrifugation, and the supernatant solution was concentrated *in vacuo* to 11 ml. 44 ml of methanol and 90 ml of ether were added. After 1 hour at 5°, the precipitate was harvested by centrifugation. The supernatant solution was concentrated to 2.5 ml *in vacuo*, readjusted to pH 7.5 with  $\text{Ca}(\text{OH})_2$ , and treated with 4 volumes of methanol and 8 volumes of ether. The precipitate was collected and pooled with the previous one. Very little keto acid remained in the supernatant solution. The calcium salt was dried under a vacuum to a glassy amorphous solid, which appeared to crystallize when scraped, but which was obviously impure. 248 mg were obtained and were found to contain 103  $\mu$ moles of the keto acid.

When a solution of the calcium salt was treated with HCl and hydroxyl-

amine, the hydroxamic acid value obtained was equivalent to about 18.5 per cent of an equivalent amount of calcium arabonate. Although the presence of arabonate could be shown by chromatography, it is probable that the actual amount of this compound was much less than 18.5 per cent, since the keto acid itself probably gives a weak hydroxamic acid test under the same conditions. Attempts to purify the compound by crystallization of the calcium salt or of the free acid or the lithium, sodium, or potassium salt did not give promising results.

*Properties of 2-Keto-3-deoxy-D-arabonate*—As stated earlier, the keto acid could be separated by paper chromatography from arabonate, but great losses were sustained. The  $R_F$  values were Solvent I, arabonate 0.29, keto acid 0.42, Solvent II, arabonate 0.34, keto acid 0.61. When sprayed with *o*-phenylenediamine reagent (6), the spots gave a yellow color which has been found to be characteristic of 2-keto-3-deoxy-D-galactonic and 2-keto-3-deoxy-6-phosphogluconic acids. The quinoxaline derivative shows a typical absorption spectrum with a maximum between 322 and 325  $m\mu$ . The extinction coefficient at 325  $m\mu$  was calculated as  $5.48 \times 10^6$  cm per mole from the data obtained by decarboxylation with ceric sulfate. The semicarbazone derivative also has a spectrum characteristic of  $\alpha$ -keto acids, with a maximum at 250  $m\mu$  (extinction coefficient  $5.94 \times 10^6$  cm per mole). A crystalline 2,4-dinitrophenylhydrazone could be obtained easily. The hydrazone is relatively insoluble in acid, but dissolves in alkali in which it has a dark reddish brown color.

Solutions of the calcium salt, decationized with Dowex 50, had an absorption spectrum similar to those of pyruvate and 2-keto-3-deoxygalactonate. They showed a strong absorption below 270  $m\mu$ . On addition of alkali, a strong absorption peak at 272  $m\mu$  appeared. This behavior is not observed with the other compound and suggests that a rearrangement of the molecule may occur, possibly with the formation of an enediol. The crude calcium salt reduces alkaline ferricyanide. From the data for ceric sulfate decarboxylation, the reducing value of the pure compound was computed to correspond to about 53 per cent of that obtained with an equimolar solution of glucose by the method of Schales and Schales (17). The compound is not decarboxylated with 4-aminoantipyrine (18), which reacts with  $\beta$  keto acids.

Since the crude salt contained arabonate, a direct demonstration of the ability of the compound to form a lactone was impossible. However, when the keto acid was separated from arabonate by paper chromatography by using Solvent II and the eluate from the region containing the substance was treated with HCl and hydroxylamine, a weak color reaction characteristic of hydroxamic acids was obtained. Although this cannot be considered as proof that the keto acid can be lactonized, it suggests such an

interpretation, especially since it has been shown that pure 2-keto-3-deoxygalactonic acid also gives a weak lactone reaction under similar conditions.

The identity of the keto acid which was obtained as the crude calcium salt with the biologically active intermediate in arabinonate oxidation was established by showing that the compound is quantitatively oxidized by enzyme preparations with the reduction of 1 mole of DPN and the production of 1 mole of pyruvic acid. In a spectrophotometric experiment, 0.31  $\mu$ mole of keto acid, based on ceric sulfate decarboxylation, was incubated with 0.5  $\mu$ mole of DPN and 0.1 ml of the enzyme solution in a total volume of 3 ml of 0.03 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloric acid buffer at pH 9.0. When 0.3  $\mu$ mole of DPN was reduced, a dilute solution of crystalline lactic dehydrogenase was added. The DPNH was rapidly and completely reoxidized, indicating that an amount of pyruvate was present equivalent to the amount of substrate oxidized. DPNH was not oxidized in the presence of lactic dehydrogenase and the original keto acid under the conditions of the experiment. In a manometric experiment, 0.2 ml of enzyme was incubated with 13.6  $\mu$ moles of the keto acid, 0.4  $\mu$ mole of DPN, and 0.1 ml of a dilute solution of DPNH oxidase in 2 ml of 0.075 M Tris-hydrochloric acid buffer, pH 9.0, in the presence of air. When 5.15  $\mu$ moles of  $O_2$  were taken up, the reaction was stopped and the solution analyzed for pyruvate and glycolate, 9.0  $\mu$ moles of pyruvic acid and 10.0  $\mu$ moles of glycolic acid were found.

The biologically active compound was partially destroyed by boiling a solution of the calcium salt for 1 minute at pH 6.8. It was also destroyed by being boiled with acid and by being boiled or by standing at room temperature at pH 9.0 in Tris buffer.

*Ceric Sulfate Decarboxylation of 2-Keto-3-deoxyarabinonate*—In the experiments described earlier, the keto acid was quantitatively estimated by oxidative decarboxylation with ceric sulfate, and the results obtained were found to be in agreement with biological assays. In addition to  $CO_2$ , the product of the primary oxidation of 2-keto-3-deoxyarabinonic acid with ceric ion in acid conditions would be expected to be the  $\gamma$ -lactone of  $\beta,\gamma$ -dihydroxybutyric acid. When reduction of ceric ion was measured, it was found that the oxidation proceeded far beyond the primary decarboxylation, especially in dilute acid. The organic products obtained under ordinary circumstances could not be identified. An attempt was therefore made to control the degradation by the slow addition of the reagent in a quantity insufficient to cause extensive oxidation. 43  $\mu$ moles of the crude calcium salt of the keto acid were dissolved in 4.2 ml of water, and 0.5 ml of a 1:1 mixture of sulfuric acid and water was added. 0.3 ml of 0.24 N solution of ceric ammonium sulfate in 2.16 N  $H_2SO_4$  was added slowly at room temperature with constant stirring. The reaction mixture was

dehydrated with an excess of anhydrous sodium sulfate and extracted with peroxide-free ether. The ether was evaporated, and the residue dissolved in a small amount of water. An aliquot of the solution was chromatographed on paper with Solvent III. The principal spot, which appeared when the chromatograms were sprayed with either alkaline silver nitrate or with the reagents for detecting lactones, corresponded in position to the  $\gamma$ -lactone of  $\beta,\gamma$ -dihydroxybutyric acid. Another aliquot of the reaction mixture was treated with hydroxylamine and chromatographed on paper with Solvent IV. The main product, which gave the characteristic hydroxamic acid reaction when sprayed with saturated  $\text{FeCl}_3$  in water-saturated butanol, had the same  $R_F$  value as the hydroxamic acid derivative of  $\beta,\gamma$ -dihydroxybutyric lactone.

*Preparation of 2,4-Dinitrophenylhydrazone*—Since the keto acid could not be obtained in pure form, the crystalline 2,4-dinitrophenylhydrazone was subjected to analysis and degradation. 300 mg (1.47 mmoles) of potassium D-arabonate were dissolved in 20 ml of the cell-free extract containing 0.01 M phosphate buffer at pH 6.8, and the mixture was incubated for 12 hours at 30°. A conversion of approximately 80 per cent of the substrate was estimated at this time. The mixture was deproteinized with trichloroacetic acid, which was then removed by extraction with cold ether. The solution was adjusted to pH 8.5 with  $\text{Ca}(\text{OH})_2$ , and calcium phosphate was removed by centrifugation. The volume was reduced to about 15 ml under a vacuum at room temperature, and 0.3 gm of 2,4-dinitrophenylhydrazine dissolved in 2 N HCl was added. After 20 minutes at room temperature, the hydrazone was extracted with ethyl acetate. Repeated extractions were necessary. The ethyl acetate fraction was extracted several times with 10 per cent sodium carbonate solution. The carbonate solution was acidified by the dropwise addition of concentrated HCl, and a crystalline precipitate was formed. This was collected, dissolved in hot ethanol, and recrystallized in the cold after the addition of a small amount of water. Two crops of crystals were obtained from the original alcoholic solution and a third crop was obtained by adding water to the solution and extracting it with ethyl acetate. The hydrazone was extracted from the ethyl acetate with a small amount of carbonate solution, was precipitated, and recrystallized as before. All fractions melted at 163°. The total yield was 160 mg.

Some of the crystals were dried *in vacuo* over  $\text{P}_2\text{O}_5$  for 15 hours at 100° and analyzed for carbon, hydrogen, and nitrogen. Found, C 41.02 per cent, H 3.73 per cent, N 16.69 per cent. Calculated for  $\text{C}_{11}\text{H}_{12}\text{O}_8\text{N}_4$ , C 40.20 per cent, H 3.68 per cent, N 17.0 per cent.

When chromatographed on paper, by using Solvent V, the derivative had an  $R_F = 0.14$ ; pyruvic 2,4-dinitrophenylhydrazone had an  $R_F = 0.17$  under the same conditions. When sprayed with alcoholic NaOH, the 2,4-

dinitrophenylhydrazones of the keto acid turned light brown, and the hydrazone of pyruvic acid gave a dark brown color under the same conditions

*Hydrogenolysis of 2,4-Dinitrophenylhydrazones*—52.8 mg of the 2,4-dinitrophenylhydrazones were suspended in about 100 ml of water and hydrogenated in the presence of Adams' catalyst under 2 atmospheres of pressure at room temperature in a Parr hydrogenator (19). After 24 hours, the catalyst was filtered off, and the liquid was evaporated to a small volume under a vacuum at room temperature. When chromatographed on paper, by using Solvent IV, two ninhydrin-positive bands appeared. The minor, more rapidly moving component, which represented approximately 20 per cent of the total ninhydrin-positive substance, could not be identified by degradation to known compounds. From the ninhydrin reaction (20), the quantity of the major component was estimated to be 107  $\mu$ moles of  $\alpha$ -amino acid, and represented approximately 66 per cent of the theoretical yield from the hydrazone. This material was rechromatographed with Solvent IV, in which it was found to have an  $R_F = 0.19$ , slightly lower than that of glycine ( $R_F = 0.20$ ). It was then chromatographed with Solvent VI. Two ninhydrin-positive bands of equal intensity appeared. The faster moving component had an  $R_F = 0.33$ , equal to that of proline, while the slower moving one had an  $R_F = 0.21$ , almost identical with the  $R_F$  of glycine. Both gave a blue-purple color when sprayed with 0.1 per cent ninhydrin in 95 per cent ethanol containing 0.5 per cent collidine. Two spots could also be observed with the use of Solvent VII. After elution, the two compounds retained their chromatographic behavior with Solvents VI and VII but were indistinguishable with Solvent IV. Each of the two amino acids was subjected to degradations, which involved the initial oxidation with periodate. As will be seen from the following experiments, the products of cleavage of both compounds were found to be formaldehyde and the  $\gamma$ -semialdehyde of aspartic acid.

*Degradation of  $\alpha$ -Amino Acids*—3  $\mu$ moles of each amino acid were separately treated with 5  $\mu$ moles of sodium periodate in a total volume of 1 ml of dilute  $H_2SO_4$ . After incubation for 1 hour at 37°, 0.5 ml of 0.02 M sodium arsenite solution was added, and the pH was brought to 8.0 with solid sodium bicarbonate. 0.05 ml aliquots of these solutions were removed and analyzed for formaldehyde. In both cases, approximately 1  $\mu$ mole of formaldehyde was found per micromole of amino acid decomposed (the actual values were 1.1 and 1.05). The remainders of the periodate-treated solutions were treated in each case with 10 mg of sodium borohydride for 1 hour at 37°. The excess borohydride was decomposed by the addition of enough acetic acid to lower the pH to approximately 4.0. The liquid was then passed through a small column of Dowex 50 resin in the H form to absorb the amino acid formed in the treatment. The resin

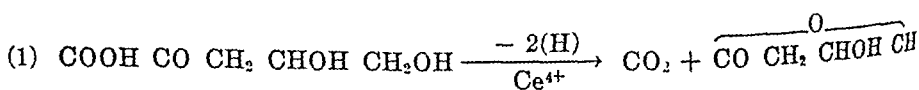


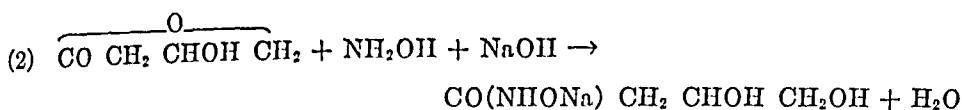
was washed with water until the eluate was neutral, and the amino acid was eluted with *N* ammonium hydroxide. The fractions which gave a ninhydrin-positive reaction were concentrated and chromatographed by using Solvents IV, VI, VII, VIII, IX, and X. From both reaction mixtures, only a single ninhydrin-reacting compound could be detected after chromatography with each solvent. In all cases this compound had the characteristic  $R_F$  and blue-purple color of homoserine when sprayed with the ninhydrin-collidine spray.

Next, the two amino acids were degraded by using a slight modification of the periodate-permanganate method of Lemieux and von Rudloff (21). 3  $\mu$ moles of each amino acid were treated with 6  $\mu$ moles of periodate in a final volume of 1.2 ml of dilute  $H_2SO_4$  for 1 hour at 37°. The pH was brought to 8.0 by the addition of solid sodium bicarbonate and 0.1 ml of 0.001 *N* potassium permanganate was added. After 15 minutes at room temperature, the liquid was passed through Dowex 50 resin, and the resulting amino acid was eluted as in the previous experiment. The ninhydrin-positive fractions were chromatographed with Solvents IV, VI, VII, VIII, IX, and X. Both reaction mixtures yielded the same single ninhydrin-positive compound. On all the chromatograms, this compound had the  $R_F$  value and the characteristic blue color of aspartic acid.

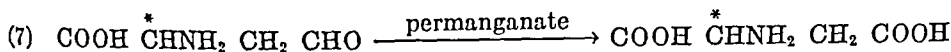
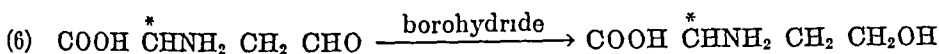
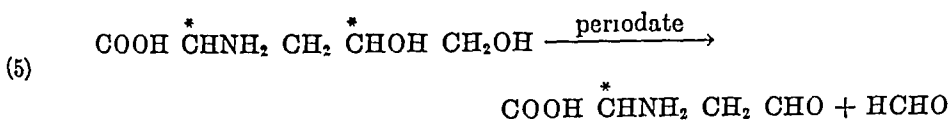
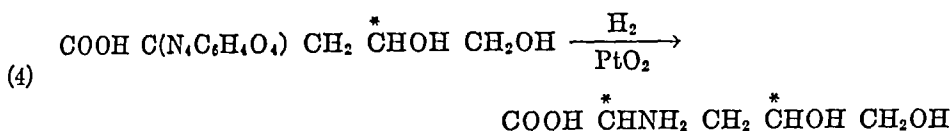
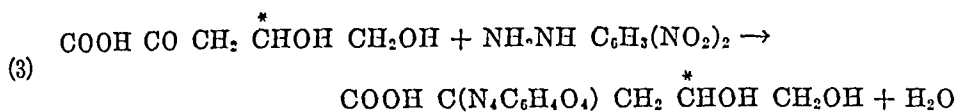
#### DISCUSSION

All of the reactions which have been described are consistent with the view that the new compound is an  $\alpha,\beta$ -dehydrated *D*-arabonic acid, having the 2-keto-3-deoxy structure which has been found in analogous compounds derived from *D*-galactonic and from 6-phosphogluconic acids (1, 7). The  $\alpha$ -keto configuration is shown by the oxidative decarboxylation with ceric sulfate, by the reaction with *o*-phenylenediamine, to give a product which has an absorption spectrum typical of quinoxaline derivatives, by the formation of a derivative with semicarbazide which has a characteristic spectrum of  $\alpha$ -keto acid semicarbazones, and by the formation of  $\alpha$ -amino acids in the hydrogenolytic decomposition of the 2,4-dinitrophenylhydrazone. The occurrence of two adjacent hydroxyl groups on the 2 terminal carbon atoms is demonstrated by the rapid production of formaldehyde in the reaction of periodate with the amino acid derivatives. The configuration of the last 4 carbon atoms is supported by the demonstration that the controlled ceric oxidation of the compound yields a product indistinguishable from  $\beta,\gamma$ -dihydroxybutyric lactone. The reactions used in this degradation can be summarized as follows:





The configuration of the first 4 carbon atoms is further demonstrated by the degradation of the 2,4-dinitrophenylhydrazine. The reactions which were used are represented by the following equations, in which the asymmetric carbon atoms are marked with an asterisk



It will be seen that a new point of asymmetry is introduced by the hydrogenolysis of the 2,4-dinitrophenylhydrazine (Reaction 4), since both D- and L-amino acids are formed in the reaction. Actually, two pairs of optical isomers of the dihydroxyamino acid are possible, and the production of one member of each pair can be expected from either of the two possible isomers of the keto acid. This explains the demonstration that two different amino acids could be separated by chromatography after Reaction 4. Since only 1 asymmetric carbon remains after Reaction 5, the products obtained in Reactions 6 and 7 are stereoisomeric and indistinguishable by chromatography.

None of the degradations which have been used establishes the configuration of C<sub>4</sub>. The stereochemistry of the 4th carbon atom, however, can be assumed to remain unchanged during the dehydration of D-arabonic acid, since this atom is probably not involved in the reaction. This assumption is supported by the observation that the analogous dehydrations of 6-phosphogluconic acid and of galactonic acid by different enzymes of the same organism do not cause inversions at C<sub>4</sub>. In the case of galactonic

acid, the evidence is particularly good, since the biologically active product of the enzymatic dehydration has been found to be identical with the chemically prepared derivative of metasaccharin but not of glucometasaccharin

Little can be said about the possible ring structures of the keto acid, except that the substance can, apparently, be lactonized under acidic conditions. It seems safe to conclude, however, that the compound is 2-keto-3-deoxy-D-arabonic acid. A description of the enzymatic steps which are involved in the production of the keto acid from arabinose and its oxidative decomposition to pyruvic and glycolic acids will be published elsewhere.

### SUMMARY

A compound which is produced from D-arabonic acid by enzyme preparations of *Pseudomonas saccharophila*, and which can be oxidized to pyruvic and glycolic acids, has been isolated as a crude calcium salt and as a crystalline 2,4-dinitrophenylhydrazone. As a result of various degradation procedures, the compound is believed to be 2-keto-3-deoxy-D-arabonic acid.

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# PARTICIPATION OF CYTIDINE COENZYMES IN THE METABOLISM OF CHOLINE BY SEMINAL VESICLE\*

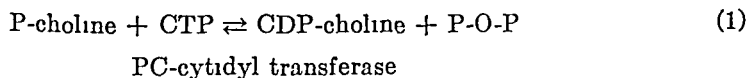
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In 1895 Florence (1) found that a brown crystalline mass is formed if a solution of iodine in potassium iodide is added to semen. Subsequent investigations by Bocarius (2) and by Staněk (3) showed that free choline present in semen was responsible for the formation of this material. It is now well established that semen is one of the richest sources of choline in animal tissues (4). The careful studies of Kahane and Lévy (5) demonstrated that human semen contains virtually no free choline immediately after ejaculation, but that large amounts of free choline are formed if the semen is allowed to stand. Further experiments by these investigators (6, 7) made it clear that the precursor of free choline in aged semen is L- $\alpha$ -glycerophosphorylcholine (GPC<sup>1</sup>). It has been reported that this substance is present in high concentrations in the secretions of the seminal vesicle, but not of other accessory reproductive glands, of a number of species (7, 8), and that it is converted rapidly to free choline by enzymes in prostatic secretion (8, 9).

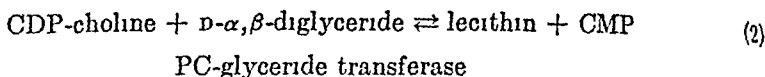
The recent discovery of the central role played by cytidine diphosphate choline in the biosynthesis of lecithin (10-12) prompted a study of the participation of this coenzyme in the synthesis of lecithin in seminal vesicle tissue, since GPC is a constituent of the lecithin molecule. The entry of P-choline as a unit into lecithin in liver can be accounted for by two reactions (12)



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† Scholar in Cancer Research of the American Cancer Society.

<sup>1</sup> The following abbreviations are employed: GPC = L- $\alpha$ -glycerophosphorylcholine, P-choline = phosphorylcholine,  $\alpha$ -GP = DL- $\alpha$ -glycerophosphate, ATP = adenosine triphosphate, AMP = adenosine-5'-phosphate, ITP = inosine triphosphate, UTP = uridine triphosphate, CTP = cytidine triphosphate, CDP-choline = cytidine diphosphate choline, CoA = coenzyme A, P-O-P = inorganic pyrophosphate, ADP-choline = adenosine diphosphate choline, Tris = tris(hydroxymethyl)aminomethane, DPN<sup>+</sup> = diphosphopyridine nucleotide, CMP = cytidine-5'-phosphate.



This paper will describe the PC-cytidyl transferase and the PC-glyceride transferase of seminal vesicle tissue. The relationship of these reactions to the secretion of GPC by seminal vesicle is discussed.

### *Materials and Methods*

The seminal vesicles were removed from sexually mature rats under ether anesthesia. Prior to excision, the coagulating glands were dissected, and the seminal vesicles ligated at the base. The seminal vesicle secretion was removed by manual expression. Homogenates were prepared, after mincing the tissue with scissors, in an all-glass apparatus immersed in an ice-salt bath. Cytoplasmic particles were isolated from 10 per cent homogenates prepared in 0.25 M sucrose. Nuclei and cell debris were removed by centrifugation at  $600 \times g$  for 5 minutes. The cytoplasmic particles were isolated from the resulting supernatant fluid by centrifugation at  $14,000 \times g$  for 20 minutes and washed once with sucrose.

The synthesis of CDP-choline and ADP-choline from P-choline-1,2- $^{14}\text{C}$  is described elsewhere (12, 13). GPC was prepared from commercial animal lecithin by hydrolysis with mercuric chloride by a procedure similar to that recently described (14). The choline-N-P ratio of the sample used was 1.0095/0.96. The product was chromatographed on paper with an  $R_f$  of 0.89 in the phenol-ammonia solvent of Dawson (15) and with an  $R_f$  of 0.48 in 75 per cent ethanol-0.05 M Tris buffer of pH 9.4. Cytidine and adenine nucleotides, and CoA of approximately 70 per cent purity, were purchased from the Pabst Laboratories. DPN $^{+}$  was obtained commercially; it was 86 per cent pure when assayed enzymatically (16).  $\alpha$ -GP was generously donated by Dr. Jean Sicé. A mixture of D- $\alpha,\beta$ -diglycerides was prepared from purified egg lecithin (17) by the action of the lecithinase D present in *Clostridium perfringens* type A toxin (18).

Choline was determined according to Glick (19) and phosphorus by the method of Gomori (20). Total nitrogen was estimated either with Nessler's reagent after digestion with sulfuric acid containing sodium sulfate and copper selenite (21) or by the Kjeldahl procedure.

The concentration of GPC in seminal vesicle tissue or seminal vesicle secretion was determined as follows. The tissue was homogenized in 66 per cent ethanol immediately after it was removed from the animal. The homogenate was centrifuged and the precipitate was washed with 66 per cent ethanol. After removal of ethanol under reduced pressure, the aqueous extract was extracted with chloroform. Traces of chloroform remaining in the aqueous phase were removed by aeration. The extract was deionized by passing over columns of Amberlite MB-1 resin and total

P was estimated in the deionized extract. Recoveries of added GPC were greater than 90 per cent by this procedure. Paper chromatography of these deionized extracts in 75 per cent ethanol-0.05 M Tris buffer of pH 9.4 showed a single phosphorus-containing spot which migrated to exactly the same position ( $R_F = 0.48$ ) as an authentic sample of GPC, showing that GPC was the only phosphorus-containing compound present in significant amounts in such deionized extracts of seminal vesicle.

The extraction of labeled lipides from enzyme incubation mixtures and methods for the removal of contaminating unreacted radioactive precursors are described by Kennedy and Weiss (12, 22). The breakdown of CDP-choline was measured by quantitative adsorption of this coenzyme upon charcoal by a procedure similar to that of Crane and Lipmann (23). The reaction was terminated by placing the tubes in a boiling water bath for 5 minutes and the charcoal was added after cooling. Neither free choline nor *P*-choline was adsorbed upon charcoal under the conditions used. The zero time control values depicted in Experiments 1 and 2 of Table IV show that, even upon the addition of adenine nucleotides to the reaction mixture, virtually all the added CDP-choline was adsorbed upon the charcoal.

Measurements of radioactivity were made with dried samples under conditions of negligible self-adsorption.

Castrations were performed via the abdominal route under ether anesthesia. Sham operations consisted of an abdominal incision. Testosterone propionate was administered in sesame oil by subcutaneous injection. The control animals received the same volume of sesame oil.

## Results

### *L- $\alpha$ -Glycerophosphorylcholine in Seminal Vesicle*

In confirmation of the findings of other investigators (7, 8), large amounts of GPC were found to be present in the seminal vesicles of sexually mature rats. Table I shows that this substance is confined largely to the seminal vesicle secretion, only small amounts of GPC were present in tissue freed from secretion. The levels of GPC in seminal vesicle were only 20 per cent of normal 6 days after castration, and the effect of castration was entirely reversed by the administration of testosterone propionate. Negligible amounts of GPC were found in the ventral prostate glands of normal, sexually mature rats.

### *Enzymatic Conversion of CDP-Choline to Lecithin*

Homogenates of seminal vesicle prepared in 0.15 M KCl-0.02 M sodium phosphate of pH 7.4, when incubated in the presence of magnesium ions, converted CDP-choline to lipide material at extremely low rates. However, the addition of ATP to a final concentration of 0.005 M or greater

permitted a rapid incorporation of  $C^{14}$  into the lipides of these preparations. Dialysis of the homogenates against the homogenization medium for 24 hours at  $2^\circ$  did not result in loss of activity. Table II shows that, in the presence of ATP, the entry of  $C^{14}$  from CDP-choline into lipid material was essentially unchanged by the addition of CoA, glycerol,  $\alpha$ -GP, GPC, choline, P-choline, and inorganic phosphate. Magnesium ions, however, were essential for this reaction. In a number of experiments the incubations were conducted in the presence of 0.004 M GPC and of 0.0077 M ATP, and the GPC was isolated from the acid-soluble fraction at the end of the experiment. As much as 65 per cent of the added GPC was recovered after incubation for 1 hour at  $37^\circ$ . Even upon the further addition of glycerol or  $\alpha$ -GP to the incubation mixture, the GPC isolated at the end of

TABLE I  
*L- $\alpha$ -Glycerophosphorylcholine in Rat Seminal Vesicle*

Group	No. of rats	Gland + secretion	Secretion	Washed gland
Operated upon by sham	8	14.6 (7.4-20.1)	24.6 (20.7-29.9)	5.4 (3.7-7.0)
Castrated 6 days	4	2.9 (1.9-4.0)		
“ 6 “ treated with androgen	4	16.1 (14.2-18.6)		

All GPC concentration expressed in terms of micromoles per gm. fresh weight. Androgen-treated animals received 1 mg. of testosterone propionate per day from the time of operation until their sacrifice. The figures in parentheses represent the range of values observed.

the experiment was devoid of radioactivity despite the entry of as much as 40  $\mu$ moles of choline- $C^{14}$  from CDP-choline into the lipid fraction.

The labeled lipid formed from CDP-choline in the presence of ATP appeared to consist mainly of lecithin. A carbon tetrachloride extract of the labeled lipides, previously washed with aqueous buffers (22), was evaporated to dryness and hydrolyzed in 0.5 N KOH for 16 hours at  $37^\circ$  in the presence of carrier choline. At the end of the incubation, casein was added, and trichloroacetic acid was added to a final concentration of 5 per cent. Insoluble material, containing unhydrolyzed lipid, was removed by centrifugation. Excess of a solution of ammonium reineckate in methanol was added to the aqueous extract and the resulting crystals of choline reineckate were harvested and washed three times with *n*-propanol. The crystals were dissolved in acetone and their radioactivity was determined. Virtually all of the radioactivity in the initial lipid extract could be accounted for as free choline after this treatment, showing that the labeled

lipides were labile to alkali in a manner similar to lecithin. In another experiment the washed lipides were hydrolyzed with dilute base according to Dawson (15) and the water-soluble products were separated by paper chromatography. By using the phenol-ammonia solvent of Dawson (15), a single radioactive spot was obtained which migrated to the same position ( $R_f$  0.89) as GPC.

TABLE II  
*Incorporation of CDP-Choline into Seminal Vesicle Lipides*

Experiment No	Additions	Choline incorporated into lipides
		<i>μmoles</i>
1	Nil	0.8
	ATP (0.0077 M)	20.8
	" + glycerol (0.0038 M)	19.6
	" + " + GPC (0.0021 M)	23.9
	" + " + CoA (0.0002 M)	24.0
2	0 time control	0.3
	Nil	2.1
	ATP (0.00077 M)	30.4
	" omit $MgCl_2$	0.4
	" + choline (0.0038 M)	28.5
	" + P-choline (0.0038 M)	31.0
	" + inorganic P (0.0077 M)	35.0
	$\alpha$ -GP (0.0038 M)	3.6
	ATP + $\alpha$ -GP	34.1
	0 time control	0.0

Reactions carried out in a final volume of 2.6 cc containing 100  $\mu$ moles of Tris buffer of pH 8.0, 30  $\mu$ moles of  $MgCl_2$ , 0.12  $\mu$ mole of CDP-choline- $C^{14}$ , and 1 cc of homogenate in 0.15 M KCl. Incubated for 60 minutes at 37°. Homogenate equivalent to 3.0 mg of N, 3.2 mg of N added in Experiments 1 and 2, respectively.

#### *Nucleotide Stimulation of PC-Glyceride Transferase*

The conversion of CDP-choline to lecithin described above is consonant with the operation of the PC-glyceride transferase reaction, previously described in liver by Kennedy and Weiss (12). However, in seminal vesicle preparations, the PC-glyceride transferase reaction requires the further addition of ATP. The same stimulation of lecithin synthesis from CDP-choline by ATP in homogenates could be demonstrated with cytoplasmic particles isolated from seminal vesicle. Further examination of the apparent requirement for ATP showed that AMP would replace the action of the former with either cytoplasmic particles or homogenates as an enzyme source. Since no oxidizable substrates were added to the reaction mixtures, it was considered unlikely that the stimulatory effect of AMP



with washed particles could be ascribed to its phosphorylation to ATP via oxidative phosphorylation. Fig 1 shows the effect of different con

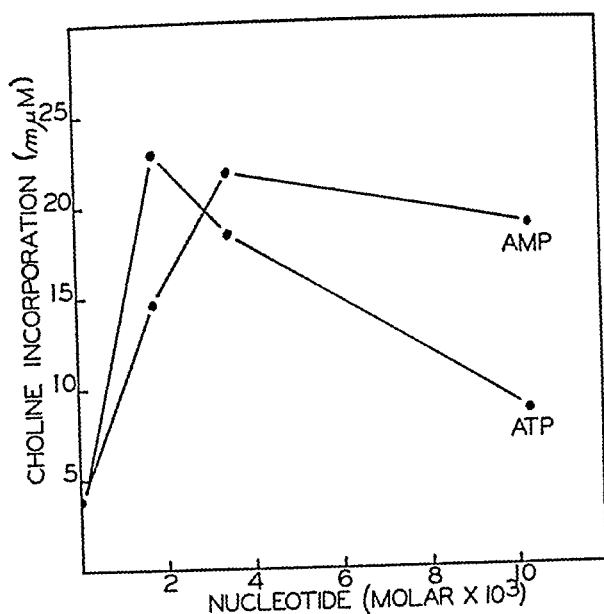


FIG 1 Stimulation of lecithin synthesis by adenine nucleotides. Each vessel contained 100  $\mu$ moles of Tris buffer of pH 7.4, 1  $\mu$ mole of  $MnCl_2$ , 75  $\mu$ moles of KCl, 10  $\mu$ moles of sodium phosphate of pH 7.4, 0.2  $\mu$ mole of CDP-choline, 250  $\mu$ moles of sucrose, and cytoplasmic particles (1.24 mg of nitrogen) in a final volume of 3.04 cc. Incubated for 60 minutes at 37°.

TABLE III  
*Nucleotide Specificity of PC-Glyceride Transferase*

Choline nucleotide added	Choline incorporated into lipides
	$\mu$ moles
CDP-choline	19.5
ADP-choline	0.05

Reaction carried out in a final volume of 2.6 cc containing 100  $\mu$ moles of Tris of pH 7.4, 1  $\mu$ mole of  $MnCl_2$ , 10  $\mu$ moles of AMP, 75  $\mu$ moles of KCl, 20  $\mu$ moles of inorganic phosphate of pH 7.4, and seminal vesicle cytoplasmic particles (1.07 mg of nitrogen). 0.2  $\mu$ mole of CDP-choline (46,000 c.p.m. per  $\mu$ mole) and 0.5  $\mu$ mole of ADP-choline (50,000 c.p.m. per  $\mu$ mole). Incubated for 60 minutes at 37°.

concentrations of AMP and ATP upon the stimulation of the PC-glyceride transferase of washed cytoplasmic particles. At low levels (0.005 M) AMP and ATP showed similar activities, but at higher concentration ATP diminished the rate of reaction. The stimulatory effect of AMP or ATP could not be replaced by adenosine (0.001 M) or adenine (0.001 M) or by lower levels (0.0002 M) of CTP, UTP, or ITP.

It was considered that the stimulatory effect of AMP or ATP might be ascribed to an intermediate formation of ADP-choline from these nucleotides and CDP-choline, and that ADP-choline would in turn act as a precursor for lecithin synthesis. However, the experiment summarized in Table III shows that chemically synthesized ADP-choline- $C^{14}$  did not act as a precursor for the formation of seminal vesicle phospholipides under conditions for which CDP-choline was highly active in this respect.

Kornberg *et al* have described an enzyme in animal (24) and plant (25) tissues which ruptures nucleotide coenzymes at the pyrophosphate linkage. Kennedy (13) has shown that potato nucleotide pyrophosphatase will split CDP-choline. Since it has been demonstrated that there is competition between various nucleotide pyrophosphates which are degraded by this enzyme (25), the effect of AMP and ATP upon the synthesis of phospholipide from CDP-choline could be explained in terms of their ability to inhibit the enzymatic breakdown of CDP-choline. The experiments summarized in Table IV show that AMP, ATP, and  $DPN^+$  inhibit the degradation of CDP-choline by seminal vesicle homogenates and various subcellular fractions. Moreover, as can be seen from Table V,  $DPN^+$ , which inhibits the breakdown of CDP-choline, also increases the entry of  $C^{14}$  from CDP-choline into the phospholipides of seminal vesicle cytoplasmic particles in the same way as AMP or ATP. It may be concluded that the ability of various adenine nucleotides to enhance lecithin synthesis from CDP-choline is due to their inhibitory influence upon the enzymatic rupture of CDP-choline. The breakdown of CDP-choline was also prevented by CMP.

#### *Properties of Seminal Vesicle PC-Glyceride Transferase*

That magnesium ions are necessary for the conversion of CDP-choline to lecithin in seminal vesicle preparations, as in liver (12), has been noted above. The stimulatory effect of 0.01 M  $MgCl_2$  could be replaced by 0.0004 M  $MnCl_2$ . Tripling this concentration of  $MnCl_2$  did not alter the activity of the enzyme. Calcium ions were found to be extremely inhibitory towards seminal vesicle PC-glyceride transferase as can be seen from Table V. In other experiments it was found that, when the molar ratio of magnesium to calcium ions was 40, the PC-glyceride transferase activity of seminal vesicle, measured in the presence of AMP, was inhibited by 78 per cent. Potassium fluoride (0.022 M) inhibited the reaction by 45 per cent. The PC-glyceride transferase activity of seminal vesicle homogenates was unaffected by acetylcholine (0.4 mg per cc) plus eserine (0.04 mg per cc) and by pilocarpine (0.4 mg per cc), these are substances which increase the rate of entry of inorganic  $P^{32}$  into the phospholipides of pancreas slices (26) and which stimulate secretion by pancreas and seminal vesicle.

TABLE IV  
Cleavage of CDP-Choline

Experiment No	Enzyme source	Additions	Incubation time	CDP choline breakdown
			min	μmoles
1	Homogenate		0	1.4
	"		10	125
	"		20	137
	"	AMP (0.0016 M)	0	0.8
	"	"	10	26.5
	"	"	20	75.5
	"	ATP (0.0016 M)	0	0.3
	"	"	10	63.0
	"	"	20	107
2	Particles		0	0.0
	"		15	261
	"	DPN (0.001 M)	0	0.0
	"	"	15	31.5
	"	ATP (0.0032 M)	0	7.5
	"	"	15	6.1
	"	AMP (0.0032 M)	0	4.1
	"	AMP	15	6.4
	"	CMP (0.0032 M)	0	4.9
	"	"	15	19.2
	"	P-O-P (0.0025 M)	15	206
	"	AMP + P-O-P	15	28.4
3	"	ATP + "	15	41.1
	"		0	0.0
	"		15	222
	"	ATP (0.002 M)	15	11.2
	"	P-O-P (0.0033 M)	15	113
	"	ATP + P-O-P	15	41.8
	"	" + " + CaCl <sub>2</sub> (0.0033 M)	15	37.3
	Nuclei		15	189
	"	ATP	15	20.1
	"	" + P-O-P	15	56.5
	Supernatant		15	94.5
	"	ATP	15	7.8
	"	" + P-O-P	15	50.0
4	"		0	1.6
	"		15	108
	"	P-O-P (0.0033 M)	15	106
	"	ATP (0.0066 M)	15	8.4
	"	" + P-O-P	15	69
	"	" + CaCl <sub>2</sub> (0.0033 M)	15	9.3
	"	" + P-O-P + CaCl <sub>2</sub>	15	69

TABLE IV—*Concluded*

Experi- ment No	Enzyme source	Additions	Incuba- tion time	CDP choline breakdown
			<i>min</i>	<i>μmoles</i>
5	Supernatant		15	93.1
	"	ATP (0.001 M)	15	4.9
	"	P-O-P (0.0033 M)	15	38.0
	"	ATP + P-O-P	15	29.0

Reactions carried out at 37° in final volumes of 1.5 to 3.0 cc containing 0.033 M Tris of pH 7.4, 0.003 M MgCl<sub>2</sub> was present. Approximately 0.25 μmole of CDP-choline added initially, except in Experiment 1 where 0.15 μmole of CDP-choline was added.

TABLE V  
*Nucleotide Stimulation of PC-Glyceride Transferase*

Additions	Choline incorporated into lipides
	<i>μmoles</i>
None	3.0
ATP	8.1
AMP	11.2
DPN	11.0
CaCl <sub>2</sub>	0.0
ATP + CaCl <sub>2</sub>	0.4

Reaction carried out in final volume of 2.0 cc containing 100 μmoles of Tris buffer of pH 7.4, 20 μmoles of MgCl<sub>2</sub>, 0.25 μmole of CDP-choline, mitochondria (0.56 mg of nitrogen) in 0.5 cc of 0.15 M KCl-0.02 M sodium phosphate, pH 7.4. 10 μmoles of each nucleotide added. Calcium chloride when added, 5 μmoles. Incubation for 60 minutes at 37°.

TABLE VI  
*Stimulation of PC-Glyceride Transferase by D-α,β-Diglyceride*

AMP	D-α,β-Diglyceride	Choline incorporated into phospholipides
		<i>μmoles</i>
—	—	2.5
—	+	2.7
+	—	10.9
+	+	18.0

Each vessel contained 100 μmoles of Tris of pH 7.4, 20 μmoles of MgCl<sub>2</sub>, 0.5 μmole of CDP-choline, 1 mg of "Tween 20," and seminal vesicle cytoplasmic particles (1.7 mg of nitrogen) in a final volume of 2.1 cc. If added, AMP 10 μmoles, D-α,β-diglyceride 2 μmoles. Incubated for 60 minutes at 37°.

In all of the experiments described above the lipid acceptor for the synthesis of lecithin from CDP-choline was present in the enzyme preparations. Kennedy and Weiss (12) have shown that special conditions *viz* the presence of detergents, are necessary for the unequivocal demonstration of a requirement for  $D\text{-}\alpha,\beta$ -diglyceride in the PC-glyceride transferase in liver mitochondria. Table VI shows that in the presence of "Tween 20" (polyethoxyethylene sorbitan monolaurate) a marked stimulation of the PC-glyceride transferase by  $D\text{-}\alpha,\beta$ -diglyceride can be demonstrated with seminal vesicle cytoplasmic particles.

### *PC-Cytidyl Transferase*

Kennedy and Weiss (12) have shown that CDP-choline is synthesized in liver by the freely reversible PC-cytidyl transferase reaction (Equation 1). The rapid breakdown of CDP-choline by seminal vesicle preparations described above, as well as the intense destruction of nucleotide and other phosphates by this tissue (27, 28), made the demonstration of the synthesis of CDP-choline from CTP and P-choline virtually impossible. However, the reverse reaction, *i.e.* the pyrophosphorolytic cleavage of CDP-choline, was readily demonstrable. Experiments 2 to 5 in Table IV show that when either AMP or ATP was added to circumvent the cleavage of CDP-choline, the further addition of P-O-P caused a marked increase in the rate of breakdown of the cytidine coenzyme. It may be noted that, in the absence of added adenine nucleotides, P-O-P itself depressed the hydrolytic breakdown of CDP-choline to some extent. The P-O-P-dependent destruction of CDP-choline observed in the presence of AMP or ATP was unaffected by calcium ions, as is the PC-cytidyl transferase of liver (12), and in marked contrast to the PC-glyceride transferase of liver and seminal vesicle. It is concluded from these experiments that seminal vesicle tissue exhibits marked PC-cytidyl transferase activity, and that this enzyme is presumably responsible for the synthesis of CDP-choline by this tissue.

### DISCUSSION

The enzymatic pathways for the synthesis of lecithin from CDP-choline, and for the formation of the latter coenzyme, by seminal vesicle here described are similar to those already demonstrated in a number of other animal and plant tissues (12) and testify further to the ubiquitous occurrence of cytidine coenzyme-dependent reactions for the biosynthesis of lecithin. The most striking difference between seminal vesicle and liver is that in the former tissue the enzymatic degradation of CDP-choline by an enzyme similar to, or identical with, nucleotide pyrophosphatase is extremely rapid. Only in the presence of nucleotides such as AMP, ATP, or  $\text{DPN}^+$ , which inhibit the degradation of CDP-choline, can the PC-glyceride transferase reaction of seminal vesicle be demonstrated to take

place at more than negligible rates. The apparent "requirement" for adenine or pyridine nucleotides for the PC-glyceride and PC-cytidyl transferases in seminal vesicle emphasizes the caution that must be taken in attributing direct participation of such cofactors in any multienzyme sequence.

The experiments described above give little insight into the origin of the large amounts of GPC present in seminal vesicle secretion. Even in the presence of a pool of added GPC, and under conditions in which rapid synthesis of lecithin occurs, no formation of GPC from CDP-choline could be detected with or without the further addition of either glycerol or  $\alpha$ -GP. Furthermore, the entry of  $C^{14}$  from CDP-choline into lecithin was unaffected by GPC. These findings suggest that a direct formation of GPC from CDP-choline and glycerol or  $\alpha$ -GP does not take place in seminal vesicle tissue.

Recently, Schmidt *et al* (29) have reported that the livers of certain species contain considerable amounts of GPC and that the levels in this tissue, unlike those in intestine or pancreas, do not increase upon autolysis. They suggest that GPC may function as a building block for the synthesis of phospholipides. In this connection it may be mentioned that preliminary experiments in this laboratory have shown that, whereas seminal vesicle homogenates supplemented with ATP and CoA can esterify palmitate-1- $C^{14}$  to lipid material in the presence of  $\alpha$ -GP, little or no esterification of the fatty acid occurs when  $\alpha$ -GP is replaced by GPC. This suggests that seminal vesicle tissue can readily form phosphatidic acids by esterification of  $\alpha$ -GP by the pathways described by Kornberg and Pricer (30), but that under the same conditions GPC cannot be esterified to form phospholipide. Attempts to demonstrate the formation of GPC from free choline-1,2- $C^{14}$  and from P-choline by seminal vesicle preparations with the addition of ATP, CoA, and CTP, with or without fluoride present, have been uniformly unsuccessful.

Dawson (31) has shown recently that it is unlikely that GPC in rat liver is a precursor of lecithin, and that GPC probably originates from the hydrolysis of lecithin. Experiments are under way to determine whether the GPC in seminal vesicle secretion is derived from the degradation of phospholipides which contain the elements of GPC as part of their structure.

#### SUMMARY

- 1 The presence of high concentrations of L- $\alpha$ -glycerophosphorylcholine in the seminal vesicle secretion of sexually mature rats has been confirmed. The levels of this substance fall after castration and can be restored to normal by the administration of androgens.

- 2 Seminal vesicle homogenates and cytoplasmic particles catalyze the

synthesis of lecithin from cytidine diphosphate choline and D- $\alpha,\beta$ -diglyceride. Under a number of experimental conditions for which cytidine diphosphate choline acts as a precursor for the synthesis of lecithin in seminal vesicle, no formation of L- $\alpha$ -glycerophosphorylcholine from this cytidine coenzyme could be detected.

3 Homogenates of seminal vesicle, and various subcellular fractions isolated therefrom, degrade cytidine diphosphate choline rapidly. This enzymatic destruction of cytidine diphosphate choline was inhibited by various adenine and pyridine nucleotides. The enzyme responsible for the cleavage of cytidine diphosphate choline in seminal vesicle is probably identical with the nucleotide pyrophosphatase of Kornberg.

4 Evidence is presented for the occurrence in seminal vesicle of an active PC-cytidyl transferase, which catalyzes the reversible formation of cytidine diphosphate choline from cytidine triphosphate and phosphorylcholine.

5 The origin of the L- $\alpha$ -glycerophosphorylcholine in seminal vesicle secretion is discussed.

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# THE SEPARATION OF SPHINGOLIPIDES BY ADSORPTION CHROMATOGRAPHY\*

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It has been shown that the sphingolipide fraction is labeled during perfusion of monkey brains with either acetate-1-C<sup>14</sup> or octanoate-1-C<sup>14</sup> (1, 2). To ascertain the site, or sites, of labeling, it is necessary to separate and identify the components of the sphingolipide mixture. The early classical procedures for the isolation of individual sphingolipides from tissues of the central nervous system have depended essentially on differences in solubility. For example, glycosphingosides<sup>1</sup> (cerebrosides) have been separated from phosphingosides<sup>1</sup> (sphingomyelins) by means of cold and hot pyridine, respectively (4, 5), or by use of a series of chloroform-methanol solutions of graded concentrations (6). Separation of the closely related glycosphingosides, kerafin and phrenosin, has been achieved by fractional precipitation from acetone (4). Such methods are inadequate for our purposes, they involve lengthy procedures, require large amounts of material, do not give quantitative recovery of the individual sphingolipides, and do not insure the complete removal of other lipides, owing to the dissolving effect of one lipide on another.

In recent years, other techniques have been applied to the problem of lipide separation. Phosphosphingosides have been freed from glycosphingosides by adsorption of the latter on alumina (7), and from glycerol-containing lipides either by transesterification with sodium ethylate in ethanol (8) or by mild saponification with dilute aqueous alkali (9). Glycosphingosides have been freed from phospholipides either by precipitation from chloroform-methanol solution with an aqueous solution of trichloroacetic acid (10) or by passage through a column of magnesium silicate (11). Taurog *et al.* (12) separated choline-containing from non-choline-containing phospholipides of liver by adsorption on magnesium oxide. In the present study the last mentioned approach was tried first without success, about 35 per cent of the phosphorus and 43 per cent of the choline were adsorbed.

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<sup>1</sup> The nomenclature recommended by Folch and Sperry (3) is used throughout this paper.

by magnesium oxide from sphingolipide mixtures dissolved in methanol petroleum ether

Silicic acid has been used as the adsorbent in a variety of lipide fractionations Borgstrom (13) achieved the quantitative separation of phospholipides from mixed lipides by adsorption on silicic acid from chloroform solution and elution with methanol, and he fractionated mono-, di-, and triglycerides on similar columns by the use of chloroform, benzene-chloroform, and benzene solutions, respectively (14) Silicic acid columns have also been used to fractionate the plasma lipides (15), to separate fatty acids from phospholipides (16), to isolate acetal phospholipides (17), and to separate phosphatidyl ethanolamine, lecithin, and lysolecithin from a phospholipide mixture (18) The present communication adds to this list a method for the quantitative separation of small amounts of sphingolipides by chromatography on silicic acid columns

#### EXPERIMENTAL

*Materials*—Merck's silicic acid and reagent grade acetone, chloroform, and methanol were used without further purification

*Preparation of Sphingolipides*—A modification of the procedure of Carter *et al* (19) was used in isolating sphingolipides One-half of a fresh monkey brain weighing about 25 gm was homogenized with 250 ml of acetone in a Waring blender, and the suspension centrifuged The residue was treated successively with three 200 ml portions of acetone and three 200 ml portions of petroleum ether, the suspension being centrifuged after each addition of solvent In those experiments in which the brain had been perfused with a C<sup>14</sup>-labeled compound, the homogenate in acetone was first dried at low temperature *in vacuo* so that the acetone- and petroleum ether-soluble lipides could be obtained in the absence of water for other studies The residue, dried in air, from the foregoing extractions was treated with three 200 ml portions of boiling 95 per cent ethanol, and the suspension filtered with suction after each treatment The precipitate which formed when the combined ethanol extracts were chilled to 5° was filtered and washed with acetone The white powder contained 16.5 to 17.6 per cent hexose (20) and 0.81 to 1.00 per cent phosphorus (21) For purposes of comparison, sphingolipides were also isolated by the same extraction procedure from 10 pounds of lipides of beef spinal cord,<sup>2</sup> and the resulting light tan powder weighed 940 gm and contained 16.3 per cent hexose and 1.04 per cent phosphorus

All sphingolipide preparations were examined for the presence of impurities (*i e* compounds not containing sphingosine) by determination of ester

<sup>2</sup> The spinal cord lipides were obtained from Armour and Company, Chicago The author is indebted to Dr Norman S Radin for suggesting this source

groups (22), fatty aldehyde,<sup>3</sup> free cholesterol (23), and free hexose (Benedict's solution) The qualitative ninhydrin and orcinol reactions were also applied, and in some cases the sphingolipides were subjected to purification by the method of Folch *et al* (24)

*Preparation of Column*—A suspension prepared by thoroughly stirring 50 gm of silicic acid in 100 ml of chloroform-methanol (2:1, v/v) was slowly poured into a 20 × 400 mm column fitted with a fitted disk of coarse porosity, above which was a bit of glass wool Entrapment of air was avoided As solvent passed through the column, with the concomitant settling of the silicic acid, more chloroform-methanol was added until a total of 250 ml had been used When the level of solvent approached the surface of the adsorbent, chloroform was added in several portions totaling 200 ml If the column had been properly prepared, after most of the chloroform had passed through, the silicic acid was uniformly translucent If any areas of opacity remained, probably because of inadequate mixing of the silicic acid and chloroform-methanol, the column was rejected A fresh column was prepared for each determination

Attempts were made to accelerate the preparation of the column and to increase its flow rate either by mixing Hyflo Super-Cel with the silicic acid (14) or by adding the silicic acid, previously washed by decantation with chloroform-methanol, directly to the column from a chloroform suspension Both measures diminished the resolving power of the column

Significant amounts of silicic acid were carried into the effluent during the preparation of the column, washing with chloroform being continued until the effluent was free from residue If silicic acid was present in the lipid fractions, as occasionally happened despite this precaution, it was removed by treating the residue obtained on evaporation with chloroform-methanol and filtering it through sintered glass

*Operation of Column*—The last chloroform wash was allowed to flow until the surface of the adsorbent was barely exposed At this instant, the sphingolipide sample, dissolved in 10 ml of chloroform-methanol, was added with care to avoid turbulence in the adsorbent The solution was allowed to enter the column until the silicic acid surface was just exposed when 10 ml of chloroform were added After this had drained to the adsorbent surface, 50 ml of chloroform were added The column was clamped into position on a constant volume fraction collector and connected to a gradient elution apparatus similar to the one described by Busch *et al* (25) Methanol was transferred by the pressure of dried air from a reservoir containing 500 ml to a flask which contained 400 ml of chloroform, and in which the

<sup>3</sup> Aldehydes were determined by a method developed by Dr J Wittenberg and Dr S R Korey, to whom the author is indebted for details of the procedure prior to publication

solvents were mixed by magnetic stirring. The solvent was carried to the column by a glass tube through a rubber stopper which sealed the chromatographic tube. A 21 gauge needle to which was attached a short piece of rubber tubing with a pinch clamp, and which was inserted through the stopper, provided a manual pressure release valve. The eluting solvents came in contact only with glass, and the pressure was regulated to maintain a flow rate of 40 to 45 ml per hour. The eluates were collected in 5 ml fractions.

*Analytical Methods*—The eluates were combined in groups of five and analyzed for hexose, with galactose as standard (20), and phosphorus (21). The fractions which lay within a given band, as established by these determinations, were combined, concentrated under a stream of nitrogen in a water bath at 50°, transferred quantitatively to tared 10 ml volumetric flasks, and dried to constant weight. Determinations of the following were then carried out: fatty aldehyde, free amino groups, ester groups (22), hexose, phosphorus, iodine number (26), neuraminic acid by the orcinol reaction (27), choline, and nitrogen. The mercuric acetate catalyst gave erratic results and was omitted in the determination of the iodine number (28). The method used for choline determination is based on the isolation of the reneckate from butanol solution (29) and application of the Cazeneuve reaction (30) after oxidation to chromate. Nitrogen was determined by digestion with concentrated  $H_2SO_4$  in sealed tubes at 370° for 18 hours and subsequent nesslerization. Radioactive samples were counted to a standard error of 5 per cent, all the values were corrected to infinite thickness.

*Isolation of Fatty Acids*—Portions of the corresponding fractions from several chromatographic separations were obtained, evaporated under nitrogen, and hydrolyzed with 25 ml of 18 N methanolic  $H_2SO_4$  for 5 hours (31). The fatty acids and their methyl esters were extracted with several portions of petroleum ether (b p, 60–70°) totaling 40 ml, the solvent was removed by a stream of nitrogen, and the residue was saponified by refluxing it for 30 minutes with 20 ml of N NaOH in 85 per cent ethanol. The solution was acidified and the fatty acids were extracted with petroleum ether. The extract was dried over  $Na_2SO_4$ , and, after removal of the solvent, the fatty acids obtained from each band were subjected to fractional precipitation from absolute, 80 per cent, 67 per cent, and 50 per cent ethanol and recrystallized three or more times from the solvent of origin.

### Results

The analyses of the sphingolipides before chromatography indicated the absence of fatty aldehydes, free cholesterol, and free hexose, whereas the

ester group, anhydride, and orcinol reactions were positive. Application of the purification procedure of Folch *et al* (24) had no detectable effect on the analyses.

In a typical chromatographic separation of a sphingolipide preparation from monkey brain, the presence of at least seven components was demonstrated (Fig 1). Of these, four emerged from the column in well defined fractions, the smallest of which was eluted in band I by chloroform containing small amounts of methanol in the second 50 ml of effluent. Band II appeared after 175 ml of solvent had passed through the column.

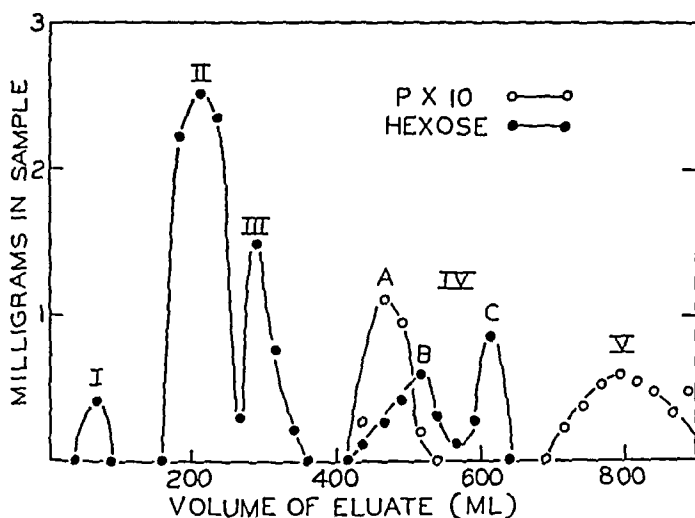


Fig 1 Chromatographic separation of 94.7 mg of a sphingolipide preparation from monkey brain. A silicic acid column and gradient elution with chloroform and methanol were used. Band I was eluted with chloroform containing small amounts of methanol. The concentration of methanol was approaching 100 per cent during the elution of band V. Each sample contained 25 ml of eluate. The phosphorus values were arbitrarily multiplied by 10 for convenience in plotting.

Although this band comprised the major fraction (Table I), its emergence was sharp and spread over a relatively small volume of effluent. Band III, the second largest fraction, followed immediately after band II and was equally sharp. There next appeared three peaks, A, B, and C, which were less well defined and could not be further resolved. They were grouped under the designation of band IV and analyzed together. Band V was most strongly adsorbed, as seen by its position and broad, plateau-like shape. It has been shown that the strength with which lipids are held by an adsorbent varies as a function of their polarity (32). During each determination, a yellow band, adsorbed at the top of the column, remained immobile during the development of the chromatogram. According to weight, the five fractions accounted for an average of 78 per cent of

the material placed on the column, but average recoveries of 94.4 and 93.4 per cent, respectively, of hexose and phosphorus were obtained. Some of the loss represented by these values may be explained by retention of material on the column, particularly in the yellow band. Also, in pooling the samples, some eluates at the extremities of the bands were omitted. It is evident that the material which was lost in these ways contained much smaller concentrations of hexose and phosphorus than the fractions which were analyzed. A sphingolipide preparation from beef spinal cord gave results (Fig. 2) which were similar to those yielded by the preparations of monkey brain. The same number of bands was obtained in the same relative

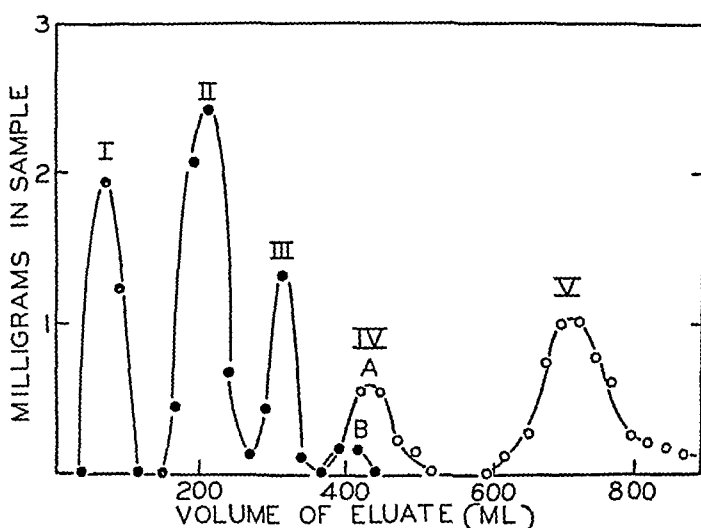


FIG. 2 Chromatographic separation of 98.0 mg. of a sphingolipide preparation from beef spinal cord. For the procedure and meaning of the characters used in plotting, see the legend to Fig. 1.

tive positions, but there were differences. Band I was much larger, band IV much smaller, and hexose Peak C was absent. Bands I to V contained 21.0, 37.6, 14.3, 7.3, and 19.8 per cent, respectively, of the total weight recovered.

In some experiments, aliquot portions of the sphingolipide preparations were chromatographed with and without prior purification by the method of Folch *et al.* (24). The results, which were unaffected by the purification, render unlikely the possibility that water-soluble substances such as hexose phosphates were present in any of the fractions.

Occasionally, the solution of sphingolipides in chloroform-methanol was turbid. Such solutions were usually filtered, but the results were the same when they were applied to the column without treatment.

Most of the data reported here were obtained on sphingolipide sample-

weighing from 60 to 100 mg. These amounts are well within the resolving power of the column. In several experiments, 200 mg samples (4 mg per gm of silicic acid) were cleanly separated, but a pronounced overlapping of bands occurred when 400 mg samples were used.

The analytical data (Table I) show that hexose was present in all bands except band V and phosphorus only in bands IV and V, and choline was absent from all bands except band V. The percentage values and molar ratios for bands I and II agree closely with the theoretical composition of

TABLE I  
*Composition of Bands Obtained from Chromatography of Monkey Brain  
Sphingolipide Mixture on Silicic Acid*

Component	Original mixture	Band No				
		I	II	III	IV*	V
Choline, %	2.46	0	0	0	0	13.84
Hexose, %	16.04	20.16	21.57	21.44	10.4 (a)	0
Nitrogen, %	2.17	1.66	1.67	3.24	3.23 (b)	3.27
Phosphorus, %	1.05	0	0	0	2.22 (c)	3.45
Choline N, molar ratio	0.13	0	0	0	0	0.51
Choline-P, " "	0.61	0	0	0	0	0.99
Hexose N, " "	0.57	1.01	1.02	0.55	0.32 (d)	0
Hexose-P, " "	2.71				0.84 (e)	0
Phosphorus-N, molar ratio	0.21	0	0	0	0.39 (f)	0.50
Ester group P, " "					0.31 (g)	0
Fatty acid, °C (m p)		84	101-102	84-85		73-74,† 52-53†
Weight, %	100.0	4.4	38.1	24.5	19.3	13.7

\* These quantities represent averages of values ranging from 1.89 to 21.5, 2.40 to 4.15, 1.95 to 2.52, 0.05 to 0.50, 0.16 to 1.66, 0.24 to 0.68, and 0 to 0.66 for (a), (b), (c), (d), (e), (f), and (g), respectively.

† Two fatty acids, melting at the points indicated, were obtained from this band.

conventional glycosphingosides. The data for band III indicate that it consisted of a glycosphingoside which contained 2 atoms of nitrogen per mole of hexose, and those for band V are in close agreement with the theoretical composition of phosphosphingosides. The widely variable data from band IV are in accord with the chromatographic findings (Figs. 1 to 3) which show that a mixture of substances was present in this fraction. The substances which reacted with orcinol and ninhydrin in the original preparation were present in band IV, these tests being negative in the other bands. The presence of neuraminic acid, indicated by the orcinol reaction, was also suggested by the observations that a brown precipitate formed when aliquots of the material in band IV were heated with 16 per



cent  $\text{H}_2\text{SO}_4$  and that the solution remained colorless with the formation of a fine, white precipitate in the presence of 0.2 N NaOH (33). In most experiments, ester groups were found in band IV in significant amounts (Table I). The other bands showed at the most a very slight ester group reaction. The average ester group-phosphorus molar ratio in band IV from beef spinal cord preparations was much smaller (0.06) than that from monkey brain sphingolipides. The average ratios of total band V to total band IV phosphorus were 1.78 and 3.03 for monkey brain and beef spinal cord preparations, respectively. In agreement with the analyses of the total sphingolipides, no fatty aldehydes were detected in any of the band

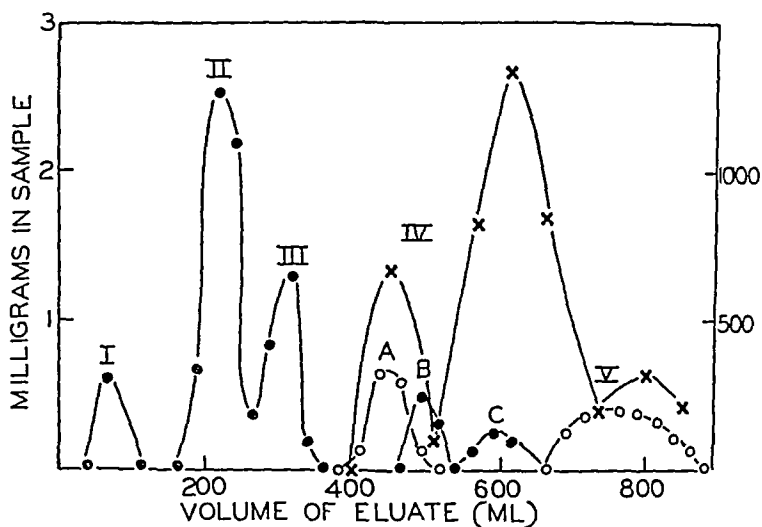


FIG 3 Chromatographic separation of 76.2 mg of a  $\text{C}^{14}$ -labeled sphingolipid preparation from monkey brain. Radioactivity in total counts per minute is shown by X, and plotted according to the scale on the right vertical axis. For the procedure and meaning of the other characters used in plotting, see the legend to Fig 1.

The average iodine number of the sphingolipide preparations was 31.8. The iodine numbers of the five bands, which were all fairly close to this value, gave no indication of the separation of a fraction with a consistently high or low degree of unsaturation. The theoretical iodine numbers of the glycosphingoside, kerafin, and the phosphosphingoside, lignoceroylsphingosylphosphorylcholine, are 31.3 and 30.5, respectively.

In an attempt to identify further the compounds present in the various fractions, the fatty acids were isolated from all of the bands except band IV. Bands I and III each yielded a fatty acid which crystallized from 67 per cent ethanol. Band II gave a fatty acid which crystallized from absolute ethanol. Two fatty acids were derived from band V, one crystallizing from 80 per cent and the other from 50 per cent ethanol. The limited amounts of these fatty acids precluded any examination of them beyond the deter

mination of their melting points (Table I) No conclusions can be drawn from such data, but the results are in accord with the assumption that the fatty acids had the following identities Bands I and III, lignoceric, band II, cerebronic, and band V, arachidic (higher melting of the two present) The reported melting points of these fatty acids are 83–84°, 100–101°, and 74–76°, respectively No identity is suggested for the acid from band V with a melting point of 52–53° Its lead salt melted at 99–101°

Labeled sphingolipide preparations from monkey brains which had been perfused with acetate-1-C<sup>14</sup> or octanoate-1-C<sup>14</sup> were purified by the procedure of Folch *et al* (24) and chromatographed In a typical experiment with sphingolipides from an acetate-perfused brain, one peak of radioactivity coincided with phosphorus peak (A) of band IV, another larger peak coincided approximately with the hexose peak (C) of band IV but overlapped band V, and a third small peak was over band V (Fig 3) Similar results were obtained with labeled sphingolipides from brains perfused with octanoate, except that a single peak of radioactivity was eluted, it coincided with the hexose peak (C) of band IV The possibility that the radioactivity of the sphingolipides was the result of contamination by the perfused labeled fatty acid was ruled out by an experiment in which labeled octanoic acid was added to unlabeled sphingolipides When this preparation was chromatographed, all the radioactivity emerged with the glycosphingoside of band I

#### DISCUSSION

The data obtained in this investigation give conclusive evidence that three glycosphingosides were separated in bands I, II, and III, and that two species of phosphosphingoside were present in band V, they give strong, though not conclusive, support to the assumption that the compounds in bands I and II are kersin and phrenosin, respectively The presence of 2 atoms of nitrogen per mole of hexose in band III indicates that it consists of a hitherto unrecognized glycosphingoside Since this compound gave no ninhydrin reaction, it is reasonable to assume that the 2nd nitrogen atom may be present as an acetylated amino group attached to the carbohydrate portion of the molecule

The data for band IV from monkey brain indicate that it contains at least one phosphorus-containing and two carbohydrate-containing compounds The positive orcinol reaction suggests that the carbohydrate was present in gangliosides, which are reported to contain no free amino group and to yield on hydrolysis 5 moles of hexose, 2 moles each of fatty acid, sphingosine, and neuraminic acid, and 1 mole of acetylated hexosamine (33) The presence in band IV of a free amino group, shown by the ninhydrin reaction, and the absence of choline remain to be explained It

would be possible to account for both findings by the assumption that the phosphorus-containing compound was a cephalin which had not been removed in the preparation of the sphingolipides. This explanation is rendered unlikely by the ester group-phosphorus molar ratios, which were zero in some experiments in which the usual amounts of phosphorus were present, and which appeared to vary to some extent with the concentration of hexose-containing material. The findings could be explained by the presence of a sphingolipide containing phosphorus, a free amino group, and no choline. These requirements would be satisfied by sphingosyl phosphate or analogues of sphingomyelin containing ethanolamine or serine such as the cephalin B of Biante (34), or possibly the alkali-stable, choline-free fraction described by Dawson (35). Compounds such as sphingosyl phosphate, psychosine, and ceramide, which may be intermediates in the synthesis of sphingolipides, have not been found in the brain, and no direct evidence for their presence was obtained in this investigation. Thannhauser and Boncoddio (36) found dipalmityl lecithin to be a contaminant of phosphosphingoside preparations. No evidence for its presence was obtained in this study, since no fraction contained both choline and significant amounts of ester groups. If it was present in the sphingolipide preparations, it was probably retained on the column.

Chemical changes have been shown to occur on various adsorbents used in chromatography (37, 38), including the isomerization of  $\beta$ -monoglyceride to the  $\alpha$  form on silicic acid (14). No indication of structural alterations was seen in the present studies, although it is possible that they occurred, particularly in the compounds of band IV. It was previously reported (2) that choline, fatty acids, and sphingosine, isolated from labeled sphingolipides, contained no radioactivity. This finding and the results of the present study suggest that the  $C^{14}$  activity of sphingolipides resides in the carbohydrate moiety. This interpretation leaves unexplained, however, the presence of activity in the phosphosphingosides of band V.

#### SUMMARY

A chromatographic method is described for the separation of the sphingolipides of the central nervous system by gradient elution with chloroform and methanol from a silicic acid column.

Sphingolipides from monkey brain and beef spinal cord gave similar chromatographic profiles, but there were considerable quantitative differences in the size of the fractions, and a carbohydrate-containing fraction, present in monkey brain preparations, was not detected in sphingolipides from beef spinal cord.

Three glycosphingosides were separated (in bands I, II, and III) in relatively pure form. Two of these (in bands I and II, respectively) appeared

to be kerasin and phienosin, the third contained 2 atoms of nitrogen per mole of hexose and a fatty acid which was probably lignoceric acid

Two species of phosphosphingoside were eluted in band V. The fatty acid in one of these was probably arachidic acid.

One of the fractions (band IV) from monkey brain sphingolipides included at least one phosphorus-containing and two carbohydrate-containing components, probably gangliosides. Possible explanations of the presence of a free amino group in, and the absence of choline from, this fraction are discussed.

Most of the radioactivity of sphingolipides, labeled by perfusion of monkey brains with acetate-1- $C^{14}$  or octanoate-1- $C^{14}$ , was found in band IV. Octanoate-1- $C^{14}$ , added to unlabeled sphingolipides, emerged with the glycosphingoside of band I.

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# SOME ENZYMES OF NUCLEOSIDE METABOLISM OF *ESCHERICHIA COLI*\*

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WITH THE TECHNICAL ASSISTANCE OF W. A. LAMONT

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We have recently presented a hypothesis of the mechanism by which the mutation rate of *Escherichia coli* is increased in the presence of methylxanthines (1). It was suggested that methylxanthines partially inhibit the synthesis of some nucleic acid intermediates, thus causing an increased probability for the utilization of a substitute compound in the synthesis of the specific polynucleotide that carries the genetic information. This hypothesis is consistent with two other findings: first, the complete absence of incorporation of radioactive methylpurines by growing cultures (2), and, second, the observed inhibition by methylpurines of the purine nucleoside phosphorylase present in extracts of this organism (1).

In the work presented here, we have extended these enzymatic studies. A single preparation of an extract of *E. coli* was assayed under a variety of conditions for its ability to attack various nucleosides. By using a single preparation, we were able to make reproducible measurements without the complication of batch to batch variation in enzymatic activity which was noted previously. The effects of a number of purine and pyrimidine compounds on the various nucleoside-splitting activities were then measured by carrying out each reaction in a suitable buffer at the appropriate pH. From this study the existence of two additional types of enzymes, a hydrolase and transribosidases, was inferred. These activities were then directly demonstrated by separations achieved by starch block electrophoresis.

The relationship of inhibition of enzyme activities to the hypothesis of mutagenesis as well as to other biological effects of these purines and pyrimidines is discussed.

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### Methods

*Preparation of Enzyme Extracts*—10 liters of medium were inoculated with *E. coli* strain B 1,5 and the culture was grown, harvested in the exponential phase of growth, ground with alumina, and extracted with 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.5, as previously described (1). The extract was centrifuged at  $25,000 \times g$  for 30 minutes, dispensed in 2 ml aliquots, and stored in the deep freeze. This extract appeared to be considerably more active than the preparations used previously.

*Substrates*—Four substrates were used in this study: hypoxanthine riboside (HXR), guanine riboside (GR), guanine deoxyriboside (GDR), and hypoxanthine deoxyriboside (HXDR). The first three compounds are available commercially, and the ribosides were purchased from the Schwarz Laboratories, Inc., and the deoxyriboside from the California Foundation for Biochemical Research. HXDR was prepared by the deamination of adenine deoxyriboside with a mammalian adenosine deaminase preparation (3), kindly supplied by Dr. F. Schlenk.<sup>1</sup>

It was necessary to use the hypoxanthine rather than the adenine derivatives because the bacterial extract contained a considerable amount of nucleoside deaminase which would have acted on adenine compounds, releasing hypoxanthine compounds that would migrate with different *R<sub>f</sub>* values and thus complicate the interpretation of the experimental results.

*Enzyme Assays*—The following basic assay system was used: 0.1 ml of enzyme, 0.2 ml of inhibitor or water for controls, 0.1 or 0.3 ml of substrate, and 0.1 ml of buffer, with an incubation period of 1 hour at 37°. The enzyme extract was diluted with water so that an optimal degree of reaction occurred in the experimental system. The dilution used was 1:4 for the measurement of the splitting of HXR and 1:10 for the other compound. Since hypoxanthine compounds are quite soluble, a sufficient amount of substrate could be contained in 0.1 ml. Guanine compounds are much less soluble, and 0.3 ml was required to supply the necessary amount. The amounts of the various substrates in the reaction system were (in micro-

<sup>1</sup> 20 mg of dry enzyme powder were added to 100 mg of adenine deoxyriboside in 20 ml of water, and the mixture was incubated at 37° for 20 hours. Although the solution was unbuffered, the pH did not change greatly during the course of the reaction (pH 6, original, pH 7.6, final). The reaction was followed by the spectrophotometric procedure of Kalckar (4). The reaction mixture was made alkaline (pH 11) and adsorbed on a Dowex 2 anion exchange column. Hypoxanthine compounds are held much more strongly than adenine compounds because of the ionization of the hydroxyl group of the hypoxanthine. Thus, by slowly decreasing the pH, adenine compounds are removed before the hypoxanthine compounds. The isolated product was pure, containing no adenine compounds and no free hypoxanthine, as assessed by paper chromatography. The yield was 97 per cent.

moles) HXR, 1.81, HXDR, 1.84, GR, 2.12, and GDR, 2.25. All inhibitor stock solutions were 25 mM. In the hypoxanthine experiments, the inhibitors were present at a final concentration of 10 mM, but in the guanine experiments, because of the additional volume of water, the concentration was 7.1 mM.

Three types of buffers were utilized. The first was a 0.5 M arsenate solution of specified pH. Such solutions give adequate buffering at pH 5 and 9 (the  $pK$  values of arsenic acid are 4.4 and 9.2), but not at pH 7.5, where careful neutralization is then required for inhibitors that are soluble only at low or high pH.

In this type of buffer, both the phosphorylase and hydrolase activities will be measured. The phosphorylase reaction is rendered irreversible by the spontaneous hydrolysis of the arsenate-1-phosphate that is formed by the enzymatic action. The second type of buffer contained no arsenate or phosphate and thus eliminated phosphorylase action; 0.5 M succinate was used at pH 5, and 0.5 M Tris at pH 7.5 and 9. The third type of buffer was a combination of arsenate and either succinate or Tris, and was 0.5 M in each component.

For each of the four substrates, measurement of the rate of splitting presents a different problem, although in all four cases we have relied mainly on the isolation and estimation of the free base by paper chromatography and ultraviolet absorption measurements. For HXR the chromatographic solvent system was *n*-butyl alcohol saturated with water-saturated boric acid (5), in this solvent system the ribose compounds do not migrate. The  $R_F$  values of various substances are indicated in Table I. This system is not satisfactory for the remaining three substrates, since guanine compounds do not migrate, and the deoxyribose derivatives in general are not well separated from the corresponding free bases. Therefore, we have employed a two-phase system of aqueous 5 per cent  $\text{Na}_2\text{HPO}_4$  overlaid with isoamyl alcohol (6), the  $R_F$  values of various compounds in this solvent system are given in Table V. It is to be noted that Carter originally reported that guanine does not migrate in this solvent system, presumably because of its very low solubility. However, it was found that if the guanine is applied to the paper while in solution, it migrates with a characteristic  $R_F$  of 0.47, although, if crystallization occurs, it does not migrate. With careful attention to this point, and use of Whatman No. 3 MM paper which has much greater capacity than does the thinner Whatman No. 1 paper, it has been possible to measure guanine production satisfactorily.

**Electrophoresis**—We have employed the starch block electrophoresis technique (7, 8) in an attempt to separate the observed activities. In our apparatus the block is 48 cm long, 5 cm wide, and 1.5 cm deep. The starch was washed in, and the electrophoresis was carried out in 0.05 M Tris



buffer, pH 7.5 Two experiments were performed, each with 2.0 ml samples of enzyme. In the first, which was run for 16½ hours at 200 volts at 3°, there was partial resolution of the enzyme activities (Fig. 1). In the

TABLE I  
*Enzymatic Splitting of Hypoxanthine Riboside*

Control*	RF†	pH 9.0			pH 7.5			pH 5		
		Arse-nate	Tris-arse-nate	Tris	Arse-nate	Tris-arse-nate	Tris	Arse-nate	Succinate arse-nate	S <sub>2</sub> O <sub>4</sub> ‡
		0.78 ± 0.02	0.69 ± 0.03	0.29 ± 0.02	0.96 ± 0.04	0.93 ± 0.03	0.39 ± 0.03	0.86 ± 0.04	0.50	0.12 ± 0.02
Addition		Per cent inhibition†								
Caffeine	0.61	50	38	12	59	56	-7	35	66	0
Theobromine	0.42	94	90	100	77	21	9	20	14	
Theophylline	0.53	57	40	0	47	58	-9	37	53	17
Carboxamide	0.25									
4,5,6-Triaminopyrimidines§	0.26		-40	-20		0			0	
6-Mercaptopurine	0.23		70	-30		70			51	
Tetramethyluric acid	0.57, ¶	15	28	3	20	35	57	24	40	11
	0.15									
Benzimidazole	0	87	47	50	20	6	22	3	-23	9
8-Azaguanine	0.05	35	6	17	19	10	0		-5	-31
2,6-Diaminopurine§	0.21		60			38			30	
Adenine	0.40	69	66	51	46	34	-15	4	8	-25
Guanine	0	89	21	9	7	11	6	-15	-71	-312
Xanthine	0	39	21	12	4	20	-4	-15	-38	-20
Hypoxanthine	0.21									
Uracil	0.37	5	-5	-42		0	-46		8	5
Thymine	0.52	-6	10	-17	5	-5	-31		-15	-100
Cytosine§	0.21		29			11			0	
5-Methylcytosine	0.29	38	6			0	-3		-6	0
5-Aminouracil	0.19	78	24	14	13	-2	-20	-5	-3	-14
4,5-Diaminouracil	0	65	25	30		-4	3		-21	-61
5-Bromouracil	0.39	12	6	3		0	-22		-12	-61
Thiouracil	0.42	8	6	56	2	16	10	24	5	0
Diazouracil	0.15, ¶	0	7	30		3	-50		-25	-121
	0.35¶									
4-(6)-Aminouracil	30	22								
2,4,5-Triamino-6-hydroxypyrimidine	0	12	9	-7	3	5	4		-7	-20

TABLE I—*Concluded*

\* Control rates are expressed in micromoles of free base produced per hour at 37° in the standard assay. The standard deviations of the controls run from day to day are given

†  $R_F$  in *n* butyl alcohol saturated with water-saturated boric acid

‡ Percentage inhibition =  $[1 - (\text{rate with inhibitor})/(\text{control rate})] \times 100$

§ The effect of these substances on the splitting of hypoxanthine riboside could not be measured by using the borate system because they migrate with hypoxanthine. Therefore, the reaction mixture was chromatographed by the  $\text{Na}_2\text{HPO}_4$  system

|| Indicates fluorescence of the compound

¶ Major component

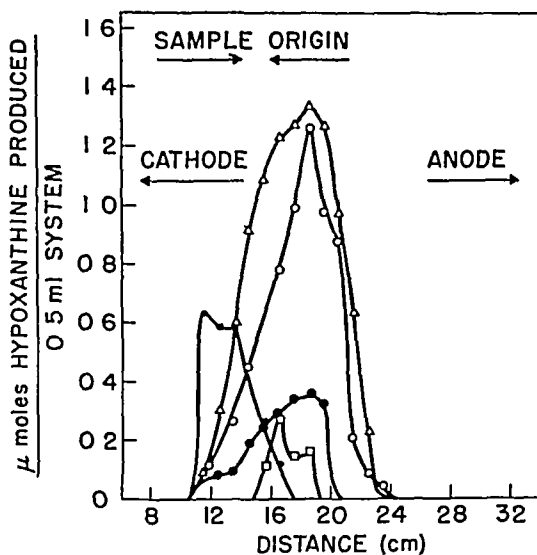


FIG 1 Run No 1 ○ riboside phosphorylase, pH 7.5, △ deoxyriboside phosphorylase, pH 5, ● transriboside-guanine, pH 5, □ transriboside-thymine, pH 5, ● hydrolase, pH 7.5. Enzyme assays were carried out as indicated in the text. For each enzyme activity, the assay was carried out at its optimal pH. Note that less enzyme solution was used for the deoxyriboside phosphorylase because of its extremely high potency. The assay period was extended 4-fold for the hydrolase and transribosidase assays. Electrophoresis conditions: 200 volts, 8 ma, 16½ hours at 3°, the pH of the electrode of vessels remained at 7.5

second the time was the same, but the voltage was increased to 500 volts, here a complete separation of the hydrolase activity was achieved, but the remaining components were not resolved. The reason for this is evident, the increased wattage brought about a situation such that the buffer capacity was locally exceeded, and this caused the pH of the anode vessel to drop to 6.9 at the end of the run. Under these conditions, the proteins which are migrating as anions become less negatively charged as they approach the anode vessel and may even change their direction of

migration. Thus, proteins of different mobilities at the initial pH may reach the same final position if the buffer capacity is exceeded.

After the electrophoresis was concluded, determinations of enzyme activity were made on 1 cm sections cut from the block. The starch sample was shaken with 2 ml of water, a quantity approximately equal to the volume of liquid contained by this amount of starch. The starch was allowed to settle and aliquots of the liquid were removed for enzymatic analysis. For all assays, a mixture of buffer, substrate and acceptor was prepared so that, when 0.2 ml (0.1 ml for the deoxyriboside phosphorylase)

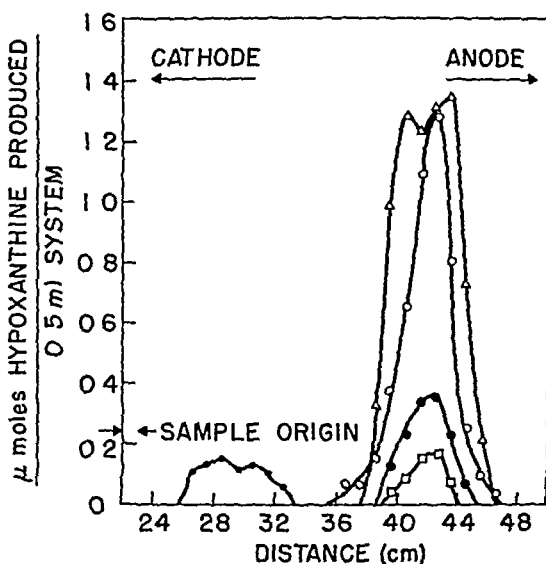


FIG 2 Run No 2 ○ riboside phosphorylase, pH 7.5, △ deoxyriboside phosphorylase, pH 5, ● transriboside-guanine, pH 5, □ transriboside-thymine, pH 5, ● hydrolase, pH 7.5. Assay conditions as described in Fig 1. Electrophoresis conditions: 500 volts, 38 ma, 16½ hours at 3°, final anode, pH 6.9, final cathode, pH 8.4.

ase analysis) was added to 0.1 ml of the mixture, the concentration of all components would be the same as that indicated above. After incubation at 37° for 1 hour, and for 4 hours in the case of the hydrolase and transribosidase analyses, aliquots were chromatographed and eluted and the extinction was measured as described. We were thus able to assay for enzyme activity, cm by cm, over the complete length of the starch block (Figs 1 and 2). In regions where no activity is indicated in the figures, no HX was produced under the specified conditions from that segment of the block.

### Results

*Splitting of HXR*—The effects of various substances on the splitting of HXR at three pH values and in different buffers are presented in Table

I The results are calculated as per cent inhibition of the control values obtained on that run, and are usually the average of two or three independent observations. It was found that usually the reproducibility of the per cent inhibition was greater than that of the control values, because variations between runs are eliminated by this method of calculation.

As indicated by the data presented, this enzyme extract catalyzes the hydrolysis of HXR. The reaction is not caused by contamination with traces of phosphate as shown by the following evidence. First, the deoxyribose compounds, which would be hydrolyzed if small amounts of phosphate were present, are not decomposed under these conditions. Second, differential inhibitory effects are observed for some of the substances tested in the two media, for example, at pH 9 diazouracil appears not to inhibit the phosphorylase activity but does inhibit the hydrolase, and caffeine and theophylline show the reverse behavior. Third, this activity can be separated from the phosphorylase.

The optimal pH of the hydrolytic reaction appears to be at pH 7.5. However, there may be two hydrolytic activities, since theobromine is a complete inhibitor at pH 9.0 but is only slightly inhibitory at pH 7.5.

In addition to hydrolase activity, another type of enzymatic activity must be assumed, since it is apparent that the addition of various purines and pyrimidines tends to *stimulate* the production of hypoxanthine from HXR. This is presumably the result of the presence of an enzyme of the transglycosidase type. The observation is of interest because it is the first demonstration of action of an enzyme upon the *N*-riboside bond as distinct from the *N*-deoxyribose bond. The presence of several distinct enzymes seems to be indicated. At pH 5, transference of the ribose between the hypoxanthine and other purines is greater than the transference to pyrimidines, the reverse is true at pH 7.5 and 9.0. It has been reported (9) that the transglycosidase activity of *E. coli* on deoxyribosides consists of two specific enzymes: a purine-purine type, and a pyrimidine-pyrimidine type. Thus, no conversion of purine deoxyriboside to pyrimidine deoxyriboside is possible in the absence of phosphate, but this is clearly not the case with the transribosidase activity.

It is of interest to know whether the transribosidases are inhibited by methylxanthines. Table II shows the inhibition of the transribosidase by caffeine at pH 5. Since the splitting of HXR in succinate buffer is unaffected by the addition of caffeine (Table I), it may be concluded that the hydrolase is uninhibited, and the effect of caffeine in the presence of guanine can be attributed to the inhibition of the transribosidase. It may be calculated from these data that the degree of inhibition of this enzyme under these conditions is 50 per cent. This experiment is not directly comparable to those in Table I, since in this experiment the concentrations of both the inhibitor and the acceptor were 5 mM (one-half the usual con-

centration) In Table III, data for the inhibition of the purine-pyrimidine transribosidase are presented, and in these experiments both the acceptor and the inhibitor were present at a concentration of 10 mM. The degree of inhibition of the transribosidase is calculated to be approximately 30 per cent at pH 7.5 and 100 per cent at pH 9.0. Thus, transribosidases are sensitive to methylxanthines. It is of interest that a number of unnatural pyrimidines are acceptors for the transribosidase. Of those tested here, 5-aminouracil, 4,5-diaminouracil, 5-biomouracil, diazouracil, and 2,4,6-

TABLE II  
*Inhibition by Caffeine of Purine-Purine Transribosidase  
Active at pH 5 in Succinate Buffer*

System	HX produced
	<i>μmole per hr</i>
HXR	0.13
" + guanine	0.51
" + " + caffeine	0.32

TABLE III  
*Inhibition by Caffeine of Purine-Pyrimidine Transribosidase  
in Tris Buffer*

System	HX produced	
	pH 7.5	pH 9.0
	<i>μmole per hr</i>	<i>μmole per hr</i>
HXR	0.357	0.285
" + caffeine	0.352	0.278
" + uracil	0.439	0.317
" + caffeine + uracil	0.393	0.272

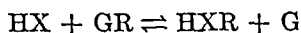
triamino-6-hydroxypyrimidine are acceptors, as is the unnatural purine, 8-azaguanine.

It is clear that the effects of the various substances on the phosphorylase are obscured to some degree by the presence of the hydrolase and transglycosidases, and one should be able to estimate the effect of a substance on the phosphorylase by comparing the results in the presence and the absence of arsenate. At pH 9, caffeine in arsenate buffer inhibits the total reaction by 50 per cent, whereas in Tris buffer the reaction is inhibited by only 12 per cent. From the reaction velocities in the inhibited and control vessels, it can be computed that the phosphorylase is inhibited by 71 per cent. Such a calculation is not valid, because there appear to be signifi-

cant buffer effects The reaction rate in arsenate is not equal to the rate in arsenate. This on arsenate-succinate buffer, moreover, the effects of some, but not all, inhibitors are different in the two types of buffer. However, it may be concluded that caffeine, theophylline, and theobromine are potent inhibitors of the phosphorylases active at neutral and alkaline pH. Adenine, guanine, and benzimidazole are also strong inhibitors. The natural pyrimidines are not inhibitors, but some derivatives of uracil are. The 5-amino and the 4,5-diamino derivatives are inhibitors, 5-bromouracil and the 2,4,5-triamino-6-hydroxypyrimidine, which are similar in structure, are much less inhibitory.

The degree of inhibition of the phosphorolytic reaction by a particular substance is considerably different at different pH values. In this particular extract there is a decrease in the percentage inhibition caused by the methyl purines at acid pH, which confirms some of our previous data (1). In general the phosphorolytic reaction appears to be more sensitive at alkaline pH.

*Stoichiometry of Transribosidase*—The identification of the transribosidase was based on the stimulation of the production of hypoxanthine from inosine by the addition of several acceptor bases in a buffer which contained neither phosphate nor arsenate. If the effect is due to a transribosidase type of enzyme, it should be possible to demonstrate stoichiometric production of the nucleoside of the acceptor base. Upon consideration of the chromatographic properties of the components of various reaction mixtures, the following system was selected



In the  $\text{Na}_2\text{HPO}_4$  +  $\alpha$ -amyl alcohol solvent system, these various components have the following  $R_F$  values: G 0.45, HX 0.57, GR 0.67, and HXR 0.74. Thus, with careful technique, one can separate all four components completely. When it is necessary to measure the amount of one component in the presence of a large amount of a neighboring component, the optical extinction is measured at two wave lengths, one, the wave length of the maximum, and the other the wave length (280  $\text{m}\mu$ ) of the maximal difference in extinction coefficients of hypoxanthine and guanine compounds.

The extent of reaction proceeding in both directions is shown in Table IV. Equilibrium is not established in either of these experiments, presumably because of inactivation of the enzymes. For each reaction, control experiments were run in which no acceptor was present, in order to test for hydrolase activity. No G was produced from GR, indicating that the hydrolase present does not attack this nucleoside. HXR is slowly attacked at this pH, and the correction for hydrolase action amounts to 0.06  $\mu\text{mole}$

at the end of the 3rd hour. Thus, it may be concluded that this activity involves the stoichiometric interchange of heterocyclic bases in *N*-ribosidic linkage, as would be expected for a transribosidase.

*Splitting of HXDR and GDR*—The results of this study are presented in Table V. The optimal pH for the splitting of adenosine deoxyriboside by extracts of *E. coli* was previously reported to be in the region of 4 to 5 (1). This was found to be the optimal pH, also, for the splitting of HXDR and GDR by the enzyme extract used here. The observation of Kalchar

TABLE IV  
*Stoichiometry of Purine-Purine Transribosidase*

(HX + GR → HXR + G)*		
Hrs	HXR, $\mu$ mole	G, $\mu$ mole
0	0.03	0.08
1	0.23	0.25
2	0.39	0.39
3	0.52	0.43

(HXR + G → HX + GR)†		
Hrs	HX, $\mu$ mole	GR, $\mu$ mole
0	0.11	
1	0.23	0.24
2	0.26	0.20
3	0.25	0.21

\* This system contained 50  $\mu$ moles of succinate buffer, pH 5.0, 0.025 ml of enzyme, 2.12  $\mu$ moles of GR, and 2.5  $\mu$ moles of HX, total volume, 0.6 ml.

† The system was the same, except that the nucleoside component was 1.81  $\mu$ moles of HXR and the acceptor was 2.0  $\mu$ moles of G.

(10) that splitting of the deoxyribose compounds, at an unspecified pH, is slower than the splitting of the ribose compounds can be explained if it is assumed that his extract was studied at a more alkaline pH. Actually, at pH 5 it can be seen that the rate of splitting of HXDR is 5 times greater than the splitting of HXR, since 2.5 times as much enzyme was used in the assay of the latter compound.

In following the effect of inhibitors on phosphorolysis, additional criteria were used. After removal of the ultraviolet-absorbing spot corresponding to the free base, the chromatograms were sprayed with the Dische reagent (11), which is specific for deoxyribose-containing compounds. With this test one is able to detect qualitatively the effect of substances, whose mi-

gation in this solvent system interferes with the determination of reaction product. By comparing the color yields at the  $R_F$  of the substrate (0.75 for HXDR and 0.66 for GDR) and of the free deoxyribose ( $R_F = 0.90$ ) in the two reaction systems, the degree of inhibition may be estimated.

In the case of GDR, yet another criterion was applicable. Guanine is very insoluble and crystallization occurs in the non-inhibited controls after several hours. If the reaction is inhibited, no crystals appear because GDR is considerably more soluble than guanine. This criterion of reaction is not satisfactory in certain cases because it was noted that in the presence of substances containing amino groups, which are not inhibitors of the reaction, no guanine crystals are formed. In the presence of these substances, 4,5,6-triaminopyrimidine, 5-aminouracil, diazouracil, and 2,4,5-triamino-6-hydroxypyrimidine, supersaturated solutions of guanine failed to crystallize, presumably because of complex formation.

In the case of adenine, hypoxanthine, and guanine compounds, trans-deoxyribosidase activity was detected by the disappearance of substrate and the appearance of a new Dische-positive spot at the appropriate  $R_F$ . Since no spot which corresponded to free deoxyribose was found, one can conclude that these substances inhibit the phosphorylase but are acceptors for the transglycosidase. Xanthine, however, does not appear to inhibit or to be an acceptor for the transglycosidase.

Neither HXDR nor GDR is split in the absence of added arsenate.

*Splitting of GR*—The results of these experiments are summarized in Table V. The enzymes catalyzing this reaction appear to be as active as those for HXR but less active than those for GDR and HXDR, they are somewhat more sensitive to the various inhibitors.

*Electrophoresis of Enzyme Extract*—The results of these experiments are shown in Figs. 1 and 2. We have corrected in all cases for the effect of hydrolase production of HX on the values for HX produced by the other activities. The correction was simple to make in the second run, since the hydrolase activity was completely separated from the other enzymatic components, in the assays for the other components, this activity appeared as a region of HX production well removed from the bulk of the activity. There was one exception to be noted. In the measurement of the deoxyriboside phosphorylase, no HX was formed in the region of the hydrolase, and it may therefore be concluded that the hydrolase is specific for ribosides.

Several conclusions may be drawn from the results presented. None of the enzymes other than the phosphorylase requires phosphate or arsenate for activity, since first it may be assumed that electrophoresis removes all traces of phosphate, and, second, in the absence of arsenate or an acceptor base, no HX is produced in regions not containing the hydrolase. The



TABLE V  
*Effect of Various Substances on Arsenolysis  
 of HXDR, GDR, and GR, at pH 5*

Control*	RF†	Substrate		
		HXDR, 1.19 μmoles per hr	GDR, 0.855 ± 0.055, μmoles per hr	GR, 0.294 μmoles per hr
Addition		Per cent inhibition‡		
Caffeine	0.70	67	75	69
Theobromine	0.66	(+ )§	27	69
Theophylline	0.56	(+++ )§	75	56
Carboxamide	0.58	(0)	-18	21
4,5,6-Triaminopyrimidine	0.20   0.47¶	6	(0)	
6-Mercaptopurine	0.39¶	55	(+++ )§	
	0.45			
	0.71			
Tetramethyluric acid	0.72	10	-5	
Benzimidazole		11	(0)	53
8-Azaguanine	0.47	(0)	(0)	
2,6-Diaminopurine	0.23	64	68	53
Adenine	0.42	(+++ )	(+++ )	
Guanine	0.47	(+++ )		
Xanthine	0.53	(0)	(+ )	
Hypoxanthine	0.58		(+++ )	
Uracil	0.72	6	13	0
Thymine	0.71	-15	9	12
Cytosine	0.71	-1	-12	-4
5-Methylcytosine	0.71	5	32	6
5-Aminouracil	0.20	-9	(0)	
	0.39			
	0.70  ¶			
4,5-Diaminouracil		18	(0)	
5-Bromouracil	0.59¶	(0)	-3	9
	0.68			
Thiouracil	0.69	-11	-3	15
Diazouracil	0.69	-4	-10	-4
4-(6)-Aminouracil	0.60	(0)	-20	6
2,4,5-Triamino-6-hydroxy- pyrimidine	0.20  ¶		27	
	0.61			
	0.75			

TABLE V—*Concluded*

\* Control rates are expressed in micromoles of free base produced per hour at 37° in the standard assay. The standard deviations of the controls are given.

†  $R_F$  in aqueous 5 per cent  $\text{Na}_2\text{HPO}_4$  overlaid with isoamyl alcohol.

‡ Percentage inhibition =  $[1 - (\text{rate with inhibitor})/(\text{control rate})] \times 100$ .

§ In these cases, production of purine could not be measured spectrophotometrically because of interference from the inhibitor. Therefore, the degree of inhibition is inferred from the Dische reaction of the paper chromatograms and in the case of guanine from the production of precipitate. Three plus signs indicate apparent complete inhibition.

|| Indicates fluorescence of the compound.

¶ Major component, *i.e.*, it contains more than 90 per cent of the  $E_{260}$ -adsorbing material. In the case of 6-mercaptopurine, the lesser component represented 20 per cent of the total  $E_{260}$ .

data indicate that at least two types of transribosidase activities are present, as suggested by the results of the inhibition studies above.

## DISCUSSION

The existence of several new enzymes is proved by these results. First, hydrolases which split the purine-ribose bond are known to occur in other organisms (12) but had been thought not to occur in *E. coli* (10). Although previously we had reported the hydrolysis of HXR in the absence of phosphate or arsenate at pH 5, but not at higher pH, we had not conclusively demonstrated the existence of a hydrolase. This extract has its greatest activity at pH 7.5 and, for the reasons mentioned above, appears to be positively identified as hydrolytic in character. The activity was probably much diminished in amount in the extracts previously studied. It has also been thought that transribosidases do not occur in *E. coli* (10), but the data of Ott and Werkman (13) are suggestive of their existence, and the results presented here conclusively demonstrate transribosidase activity. As noted above, the specificity of the transribosidase is apparently less than that reported for the transdeoxyribosidase of *E. coli* (9), which is able only to transfer the sugar from one purine to another. In this respect it resembles the transdeoxyribosidase of the other bacteria that have been studied (14). The presence of the new enzymes in the extract offers increased possibilities for the locus of action of the methylxanthines in causing mutations. The hydrolase, which is inhibited only by theobromine at alkaline pH and not by the other mutagens, is clearly not involved, but the transribosidases are inhibited by caffeine and consequently must be considered as a possible locus of action.

One purpose of the present experiments was to find out whether similar patterns of inhibition would be found with the hypoxanthine glycosides and with the guanine glycosides. The tracer data (15-17)<sup>2</sup> show that in

<sup>2</sup> Also unpublished experiments of the author.

the metabolism of this organism the pathway of biosynthesis of nucleic acid purine bifurcates into an adenine pool and a guanine pool and that these then bifurcate again to lead into the two types of nucleic acids. Therefore, it is conceivable either that the inhibition produced by the mutagen blocks the production of both purine derivatives or that one purine pool is preferentially blocked. If the latter were true, then the mutational event could be pictured as the replacement of adenine by guanine or *vice versa*. From considerations of the Watson-Crick model and the possibilities of tautomerization of the purine ring, this would be a more probable process than the temporary replacement of the purine by a pyrimidine moiety. However, the experimental data offer little to support this suggestion. Both substrates are inhibited to approximately the same extent.

In addition to studying this possibility, we were interested in trying to obtain explanations for some of the observations presented by Novick and Szilard (18). They reported that benzimidazole and tetramethyluracil are mutagens, but differ from the methylxanthines in that their mutagenic effect is only partially reversed by the addition of purine riboside. It might therefore be expected that these agents would inhibit a reaction in the synthesis of deoxyribonucleic acid at some stage beyond that of the purine riboside. What the stage may be is at present unknown, but it appears not to be one catalyzed by the purine deoxyriboside phosphorylase, since this enzyme is not inhibited by these two mutagens.

Recently, two groups of workers (19, 20) have noted that growing cultures of *E. coli* incorporate various pyrimidine analogues, including 5-bromouracil, into the nucleic acids. It is therefore of interest to find that the transribosidase will utilize 5-bromouracil as an acceptor.

Among the various substances tested, several are of pharmacological interest. The effects of natural methylxanthine on the brain, heart, and kidney are well known in man and experimental animals. 6-Mercaptopurine is being used as a drug in the treatment of cancer (21), and is apparently very toxic, which fact may be explained by its effect upon purine deoxyriboside phosphorylase. Diazouracil causes cultures of *E. coli* to form chains, *i.e.*, the cells continue to grow but cell division is inhibited (22). This substance does not appear to be a strong inhibitor of any of the reactions tested here, and this also seems to be the case for 8-azaguanine, which has carcinostatic properties and also prevents the growth of *E. coli*.

#### SUMMARY

A single enzyme extract of *Escherichia coli* was tested for nucleoside splitting enzymes. Ribosides and deoxyribosides were used as substrates, and the reaction was measured in various types of buffer with and without

aisenate, at various pH values, and in the presence of twenty-six purine and pyrimidine compounds. From this body of data, the existence of two new enzymes was inferred: first, a hydrolase which acted on inosine and, second, a transubosidase which exchanged the hypoxanthine of inosine with adenine, guanine, xanthine, thymine, 4,5-diaminouracil, 5-bromouracil, and 4,6-aminouracil. The existence of these enzymes as distinct from the nucleoside phosphorylases was further established by starch block electrophoresis. The inhibition of these various reactions by the purines and pyrimidines tested is discussed within the framework of the hypothesis that the mutagenesis produced by the methylxanthines is the consequence of the inhibition of certain enzymes of nucleoside metabolism.

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# THE MECHANISM OF THE REVERSIBLE CARBOXYLATION OF PHOSPHOENOLPYRUVATE\*

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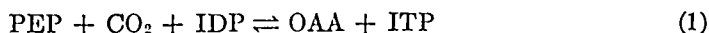
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There are two different enzyme reactions which involve a synthesis of OAA<sup>1</sup> from PEP and CO<sub>2</sub>. The reaction catalyzed by the OAA carboxylase of bird livers (1-3) as shown in equation (1) is reversible and requires as a phosphate acceptor either GDP or IDP (4). (Since there is a need



for a simple terminology to distinguish the various types of OAA carboxylases now known, the enzyme catalyzing reaction (1) will here be termed phosphoenolpyruvate carboxylase kinase or more briefly pepcarboxylase.) The reaction catalyzed by phosphoenolpyruvate carboxylase (pepcarboxylase) of spinach (5-7) and wheat germ (7) yields inorganic phosphate, as shown in equation (2), and is irreversible

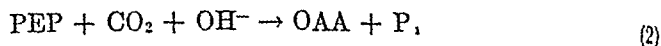
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<sup>1</sup> The abbreviations used are oxalacetate, OAA, phosphoenolpyruvate, PEP, inorganic phosphate, P<sub>i</sub>, inosine diphosphate, IDP, guanosine diphosphate, GDP, inosine triphosphate, ITP, oxidized and reduced diphosphopyridine nucleotide, DPN<sup>+</sup> and DPNH, respectively, diphosphopyridine nucleotide reduced enzymatically by 1,1'-dideuteroethanol, α-DPND. The symbol DPND is used to designate reduced diphosphopyridine nucleotide containing D in the reduced or para position of the nicotinamide ring. The prefix α indicates that the D is introduced on the side of the nicotinamide ring used by yeast alcohol dehydrogenase. The prefix β will be used for the DPND containing the D on the other side of the nicotinamide ring.



Previous experiments (8, 9) with deuterium as a tracer have shown that it is keto-OAA, and not an enol, which is formed in reaction (2), catalyzed by pepcarboxylase. The present paper describes a similar set of experiments performed with pepcarboxykinase of chicken liver. The results obtained demonstrate that, in the case of reaction (1) also, the enolic phosphopyruvate is carboxylated to give the keto and not an enol form of OAA.

These experiments involved the use of malic dehydrogenase from pig heart and showed that the H transfer caused by this enzyme is similar to that catalyzed by the malic dehydrogenase of wheat germ which has been studied previously (10).

### *Materials and Methods*

The PEP and IDP used in some of the experiments were both prepared as described previously (3). In other experiments use was made of unchromatographed PEP prepared from the Ag-Ba salt synthesized by the method of Ohlmeyer (11). Use was also made of a sample of IDP prepared from Pabst adenosine diphosphate by treatment with nitrous acid according to Lohmann (12) and Kleinzeiler (13). For this sample, identification and quantitative estimation of IDP were based on the method of Kalchauer (14).

Bovine plasma albumin was purchased from Armour and Company. DPNH was prepared from  $\text{DPN}^+$  by reduction with ethanol and alcohol dehydrogenase.  $\alpha$ -DPND was prepared from  $\text{DPN}^+$  by reduction with 1,1'-dideuteroethanol in the presence of yeast alcohol dehydrogenase (15) and contained 1 atom of D per molecule.

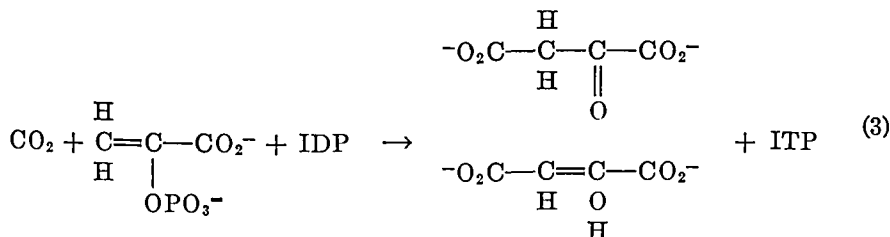
OAA carboxylase (pepcarboxykinase) was purified from chicken liver as described previously (1) until stage F was reached. This fraction was treated twice at pH 5.6 with a weight of  $\text{Ca}_3(\text{PO}_4)_2$  gel equal to the protein content of the fraction, and the gel and adsorbed material were discarded each time. The remaining supernatant fluid was lyophilized after the addition of glutathione and phosphate buffer to raise the pH to 7.0. The specific activity of pepcarboxykinase was essentially unchanged by the gel treatments, but the activity of the fumarase relative to carboxykinase activity was reduced to about one-twentieth of that of the starting material, an important consideration in the present experiments. 1 mg of powder containing about 100  $\gamma$  of protein is sufficient to exchange  $7 \times 10^{-2}$   $\mu\text{mole}$  of  $\text{C}^{14}\text{O}_2$  per minute with OAA in the exchange assay (1), if ITP is used instead of adenosine triphosphate, or to synthesize  $1.2 \times 10^{-2}$   $\mu\text{mole}$  of OAA per minute from PEP and  $\text{CO}_2$ , as measured by coupling with malic dehydrogenase (3). The comparable fumarase activity is  $2.9 \times 10^{-3}$

$\mu$ mole per minute measured according to Racker (16) Malic dehydrogenase was prepared from pig heart according to Straub (17) and had a specific activity of about 20 units per mg of protein, when a unit represents the  $\mu$ moles of DPNH oxidized per minute with OAA as the substrate

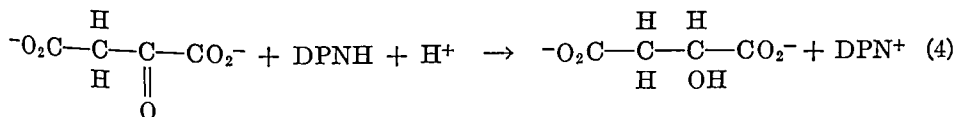
The experiments all involved a final isolation of malate as the diphenacyl derivative and its analysis for D The various reaction mixtures are described in a later section In each experiment the enzyme reaction was stopped by the addition of  $\text{H}_2\text{SO}_4$ , the protein was precipitated by tungstate, the malic acid was extracted with ether, and, in the case of the experiments carried out in  $\text{D}_2\text{O}$ , the exchangeable D was washed out with  $\text{H}_2\text{O}$  The amount of malate was determined quantitatively by chemical analysis, and an aliquot was diluted with a suitable amount of diluent L-malate This material was converted to the diphenacyl derivative which was analyzed for D All these procedures were carried out as described previously (10)

### Procedure and Results

The problem under investigation may be visualized by examination of the formulas of equation (3) The question is whether the keto or an enol



form of OAA is involved in this reaction This question was answered by carrying out the reaction in  $\text{D}_2\text{O}$  in the presence of an excess of malic dehydrogenase and DPNH Under these circumstances, the OAA formed by carboxylation is almost immediately reduced to malate by the reaction shown in equation (4) (3) If an enol of OAA is a necessary intermediate in the reaction sequence (*i.e.* equations (3) + (4)), then the malate must



contain at least 1 atom of non-exchangeable D per molecule If the malate formed by the reaction sequence contains less than 1 atom of non-exchangeable D per molecule, then an enol cannot be a necessary intermediate either of the carboxylation or of the dehydrogenase reaction Some D incorporation into malate is expected by way of the non-enzymatic keto-enol



tautomerization of OAA The amount of non-exchangeable D thus incorporated will depend on the relative rates of the keto-enol tautomerization and of the reduction of OAA It is important, therefore, that the rate of reduction of OAA be high

Since the pepcarboxykinase used in these experiments was contaminated with a small amount of fumarase, the possibility had to be considered that non-exchangeable D might be introduced into malate by way of the reaction catalyzed by fumarase To minimize the magnitude of such a side

TABLE I  
*Incorporation of D into Malate*

Experiment No	Reductant	Oxidant	Medium	Malic acid		Atom per cent excess D in diphenacyl malate	Atoms D incorporated per molecule malate†
				Formed*	Added		
				mg	mg		
1	DPNH	PEP + CO <sub>2</sub>	97% D <sub>2</sub> O	1.89	39.5	0.0135	0.054
2	"	" + "	98% "	1.01	24.6	0.022	0.101
3			98% "	1.42†	30.8	0.0035	0.014
4	α-DPNH	PEP + CO <sub>2</sub>	H <sub>2</sub> O	0.59	32.0	0.112	1.12
5	"	OAA	"	3.52	105.5	0.155	0.95
6	"	"	"	3.96	114.6	0.154	0.92

\* This is the amount diluted for preparation of diphenacyl derivative, it is somewhat less than the amount formed

† 1 atom of D per molecule of diphenacyl malate corresponds to 5.55 atom per cent excess D Sample calculation for Experiment 1 as follows  $0.0135/5.55 \times (39.5 + 1.89)/1.89 = 0.054$

‡ Amount of added malate recovered and diluted for preparation of diphenacyl derivative

reaction, the enzyme preparation used in these experiments was selected partly on the basis of its low fumarase content

The results of the experiments are given in Table I Experiments 1 and 2 show that only 0.05 and 0.10 atoms of non-exchangeable D appeared in malate when reactions (3) and (4) were conducted in sequence in a medium of D<sub>2</sub>O These are the key experiments which support the conclusion that only ketooxalacetate is involved in the reaction sequence Experiment 3 was performed as a control to determine the amount of D which could be introduced into malate by fumarase action The conditions of this experiment were identical with those of Experiment 2, except that PEP and DPNH were omitted and malate was added at the beginning of the incubation period Since the malate isolated from Experiment 3 contained considerably less non-exchangeable D than the malate isolated from

Experiments 1 and 2, the main route of introduction of non-exchangeable D into malate must be by way of the tautomerization of oxalacetate

Experiment 4 was another control experiment in which the reaction was performed in  $H_2O$ , with  $\alpha$ -DPND instead of DPNH as a reductant. The  $\alpha$ -DPND contains D in the  $\alpha$ -para position of the nicotinamide ring of DPN. If the malic dehydrogenase used in these experiments has the same (*c. a.*) steric specificity for DPN as yeast alcohol dehydrogenase, then 1 atom of non-exchangeable D per molecule should appear in malate. The value of 1.12 atoms of D per molecule found shows that this is the case. Experiments 5 and 6 are also control experiments in which OAA was reduced by  $\alpha$ -DPND in the presence of malic dehydrogenase alone. These experiments confirm the conclusion that the reaction catalyzed by muscle malic dehydrogenase involves a direct transfer of hydrogen from the  $\alpha$ -para position of the reduced nicotinamide ring of DPN to oxalacetate to give malate. In this respect, the malic dehydrogenase of heart muscle is identical with the malic dehydrogenase of wheat germ previously investigated (10).

The largest source of error in the experiments here described is the quantitative determination of malic acid in the sample used for isolation of diphenacyl malate. This source of error probably accounts for the major part of the deviation from unity of the results of Experiments 4, 5, and 6. It is apparent, however, that a 10 or 20 per cent error in the final results can have no effect whatever on the conclusions drawn.

Studies of the mechanism of the non-enzymatic decarboxylation of  $\beta$ -keto acids have shown that the keto form of the  $\beta$ -keto acid is decarboxylated to give the enol of the product (18-20). Steinberger and Westheimer (20) have shown that the catalysis of the decarboxylation of dimethylmalate by metal ions is associated with formation of a metal ion complex in which the carboxyl group  $\alpha$  to the carbonyl group is involved, and they have suggested that the metal ion-catalyzed reaction be taken as a model for the enzymatic decarboxylation of oxalacetic acid. The general requirement of oxalacetic decarboxylases for metal ions is in line with this suggestion.

The model of Steinberger and Westheimer also requires OAA to react in the keto form and pyruvate to react as the enol. The carboxylation reactions in which PEP is carboxylated to OAA have provided systems particularly suitable for testing this prediction. In these systems the enol form of pyruvate is stabilized as the phosphate. The experimental problem, therefore, is only that of distinguishing between the keto and enol form of OAA. Since the mechanism of the forward reaction is regarded as a simple reversal of the mechanism of the back-reaction, the argument is not affected

by the fact that the test system employed involves carboxylation of PEP to OAA rather than decarboxylation of OAA

The results obtained with the aid of D show unequivocally that OAA appears in the keto form, thus confirming the prediction made from the model. This applies both to the phosphoenolpyruvate carboxylase reaction studied previously (9) and to the pepcarboxykinase reaction studied in the present investigation. The fact that these reactions are quite different in some respects and are catalyzed by two distinctly different enzymes lends strong support for the validity of the basic reaction mechanism predicted by the model experiments. There are, of course, other aspects of the reaction mechanism of these enzymes which still require clarification.

#### EXPERIMENTAL

In Experiment 1, the reaction mixture contained 600  $\mu$ moles of phosphate buffer, pH 7.1, 16  $\mu$ moles of  $\text{MnCl}_2$ , 400  $\mu$ moles of  $\text{NaHCO}_3$ , 32  $\mu$ moles of PEP, 32  $\mu$ moles of IDP, 50  $\mu$ moles of DPNH, 16 mg of bovine plasma albumin, 20 units of malic dehydrogenase, and 25 mg of lyophilized carboxykinase powder in a total volume of 24 ml of 97 per cent  $\text{D}_2\text{O}$ . This mixture was incubated at room temperature for 130 minutes. Several additions of extra  $\text{NaHCO}_3$  were made during the course of the incubation in order to maintain the pH and the reaction rate. Experiment 2 was identical to Experiment 1, except that 16 mg of carboxykinase powder were used, the incubation time was 45 minutes, and the solution contained 98 per cent  $\text{D}_2\text{O}$ . Experiment 3 was performed simultaneously with Experiment 2 as a control determination of D incorporation into malate due to fumarase activity. The mixture contained 300  $\mu$ moles of phosphate buffer, pH 7.1, 8  $\mu$ moles of  $\text{MnCl}_2$ , 200  $\mu$ moles of  $\text{NaHCO}_3$ , 16  $\mu$ moles of malic acid, 10 units of malic dehydrogenase, 8 mg of bovine plasma albumin, and 8 mg of carboxykinase in a total volume of 12 ml of 98 per cent  $\text{D}_2\text{O}$ . The incubation time was 45 minutes. Experiment 4 was identical to Experiment 2, except that 50  $\mu$ moles of  $\alpha$ -DPND were used instead of DPNH, and the reaction was carried out in  $\text{H}_2\text{O}$  for 120 minutes.

In these various experiments the course of the reaction was followed by making appropriate dilutions of small aliquots of the reaction mixture and determining the optical density at 340  $\text{m}\mu$ . From these measurements, calculations were made of the amount of DPNH which had been oxidized. The amount of malate recovered in Experiments 1, 2, and 4 was approximately equivalent to 0.7 of the amount of DPNH oxidized. The excess oxidation of DPNH is assumed to be due largely to the pyruvate and to the lactic dehydrogenase which contaminated the preparations (1).

The reaction mixture of Experiment 5 contained 500  $\mu$ moles of phosphate buffer, pH 7.4, 40  $\mu$ moles of OAA, 80  $\mu$ moles of KOH, and 40  $\mu$ moles of  $\alpha$ -DPND. The mixture was incubated at room temperature for 70

minutes Experiment 6 was identical to Experiment 5, except that a different sample of  $\alpha$ -DPND was used

To prepare solutions of PEP and IDP, the salts of these compounds were suspended in a small volume of  $D_2O$  and dissolved by the addition of a very small amount of concentrated nitric acid The Ag and Ba were removed by the addition of a slight excess of NaCl and  $Na_2SO_4$ , respectively These salts were added as solutions in  $D_2O$  The PEP and IDP solutions were neutralized with solid  $Na_2CO_3$  Malic dehydrogenase was added in an  $H_2O$  solution of 120 units per ml The other constituents were added as solids

#### SUMMARY

Deuterium has been used as a tracer to show that the enzymatic addition of  $CO_2$  to phosphoenolpyruvate in the presence of inosine diphosphate, as catalyzed by the oxalacetate (OAA) carboxylase (or pepcarboxykinase) of bird liver, gives the keto, and not an enol, form of OAA These experiments also show that malic dehydrogenase of heart muscle is identical with malic dehydrogenase of wheat germ, previously studied, in the sense that both enzymes cause a direct transfer of hydrogen from reduced diphosphopyridine nucleotide (DPN) to the keto form of OAA, and the steric specificity of this transfer for DPN is identical with that shown by yeast alcohol dehydrogenase

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# A SPECIFIC SPECTROPHOTOMETRIC ASSAY FOR FLAVIN ADENINE DINUCLEOTIDE\*

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During the course of the development of a method for the chemical synthesis and large scale preparation of flavin adenine dinucleotide (FAD), it was desirable to have a rapid, yet specific, assay for the dinucleotide. A spectrophotometric method was sought, as the manometric technique of Warburg and Christian (1) with use of the D-amino acid oxidase was too cumbersome for a large number of routine assays. It was found that FAD could be determined from the rate of formation of the leuco-2,3',6-trichlorophenol-indophenol dye with the D-amino acid oxidase system. Although the rate of reduction of the dye was proportional to the concentration of FAD, the reaction proceeded too slowly for use as a quantitative assay.

In the identification of FAD as the prosthetic group of the reduced triphosphopyridine nucleotide (TPNH)-cytochrome *c* reductase of pig liver, Horecker (2) employed a spectrophotometric method by using D-amino acid oxidase coupled to the lactic dehydrogenase (LDH) system. His report, however, did not include any data on the quantitative measurement of the dinucleotide or the details of the technique. It will be shown that the method proved to be extremely efficacious in the rapid, quantitative determination of FAD. This paper is concerned with the presentation of the details of the method and its applicability in the determination of FAD in tissue extracts as well as in the detection of D-amino acid oxidase activity itself.

## Materials and Methods

*Coenzymes and Other Materials*—Diphosphopyridine nucleotide (DPN) of 90 per cent purity was obtained from the Pabst Laboratories. Reduced DPN (DPNH) was prepared enzymatically by the method of Pullman,

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Colowick, and Kaplan (3) FAD of 84 per cent purity and synthetic flavin mononucleotide (FMN) were generously supplied by the Pabst Laboratories and the Sigma Chemical Company, respectively. The concentrations of FAD and FMN were determined from their extinction coefficients at 450  $m\mu$  ( $1.13 \times 10^7$  and  $1.22 \times 10^7$  sq cm per mole, respectively (4)). DL-Alanine was obtained from the Distillation Products Industries, a division of Eastman Kodak Company, Rochester.

*Enzymes*—D-Amino acid oxidase, crystalline catalase, and crystallized skeletal muscle LDH were obtained from the Worthington Biochemical Corporation, the D-amino acid oxidase was supplied as an acetone powder of pig kidney cortex, the enzyme was partially purified and the apooxidase prepared as described in the section on "Results" by a modification of the procedure of Straub (5).

*Preparation of Tissue Extracts*—Visceral organs from normal rats were minced and homogenized in water (15 ml per gm of fresh tissue) in ice cold Ten Broeck homogenizers. The homogenate was diluted with an equal volume of 0.01 M phosphate buffer, pH 7.5, placed in a boiling water bath for 5 minutes, cooled rapidly, and centrifuged. A suitable aliquot of the clear supernatant fluid was assayed directly according to the method described below.

Subcellular fractions were prepared from rat liver, perfused *in situ* and homogenized with 10 ml of 0.25 M sucrose solution per gm of fresh tissue, by differential centrifugation according to a modification<sup>1</sup> of the method of Schneider and Hogeboom (6). Each fraction was suspended in 0.25 M sucrose, diluted with 0.01 M phosphate buffer, pH 7.5, and extracted in a boiling water bath as before, the clear supernatant fluid obtained by centrifugation was assayed as described below.

Except when indicated otherwise, microorganisms were extracted by being boiled with 20 to 30 volumes of 0.01 M phosphate buffer, pH 7.5.

*Measurements*—All spectrophotometric measurements were performed with a Beckman model DU spectrophotometer with 3.0 ml cuvettes with a light path of 1.0 cm. All the reactions were run at room temperature, and DPNH oxidation was determined by the decrease in optical density at 340  $m\mu$ . Fluorometric determinations were made according to the method of Bessey, Lowry, and Love (7), adapted to the Coleman electronic photofluorometer, model No. 12B, with filters B-2 and PC-2. FAD was determined from the increase in fluorescence after hydrolysis in 10 per cent trichloroacetic acid at 100° for 30 minutes.

### Results

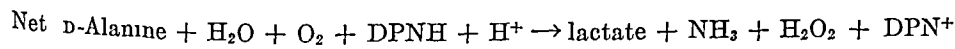
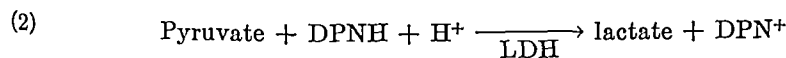
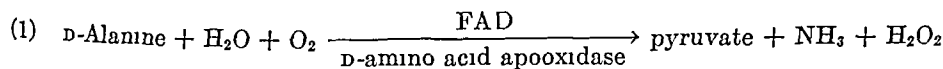
*Preparation of D-Amino Acid Apooxidase*—A number of methods have been reported for the preparation of the D-amino acid apooxidase, most of

<sup>1</sup> Jacobson, K. B., personal communication.

which are essentially modifications of the acid-ammonium sulfate precipitation of Warburg and Christian (1). Although this method results in preparations quite free of FAD, the apooxidase loses activity rapidly even on storage at  $-20^{\circ}$ . It was desirable, therefore, to use the milder procedure of Straub (5) in an effort to preserve the activity of the apoenzyme. The following is a modification of Straub's procedure which was adopted for use in all experiments cited.

The acetone powder of kidney cortex was extracted with 20 volumes of water with stirring at  $0^{\circ}$  for 10 minutes. 10 mg (dry weight) of tricalcium phosphate gel were added to each ml of the above extract to adsorb contaminating proteins, the mixture was again stirred as before and centrifuged. The precipitate was discarded, and the supernatant fluid containing the D-amino acid oxidase was precipitated by the addition of solid ammonium sulfate (22 gm per 100 ml of clear supernatant fluid). The suspension obtained was stirred at room temperature for several hours and then centrifuged. The supernatant fluid was discarded, and the precipitate was dissolved in distilled water and reprecipitated by dialysis at room temperature<sup>2</sup> against an ammonium sulfate solution (25 gm per 100 ml of water) adjusted to pH 6 to 7. Dialysis was continued with constant stirring of the precipitate within the bag. Precipitation and extended dialysis were repeated at intervals over a period of about 48 hours until an aliquot showed low activity when tested in the assay system with no FAD added. Finally the enzyme suspension was centrifuged, and the precipitate was dissolved in 0.01 M pyrophosphate buffer, pH 6.6 (10 ml per gm of original starting material). When the precipitate did not completely dissolve, the suspension was cleared by centrifugation, and the supernatant fluid containing the apoenzyme was collected. When stored in small quantities at  $-20^{\circ}$ , such preparations were stable for at least a month, with little if any loss in activity. Some preparations were found to lose no activity even after storage for 2 months at  $-20^{\circ}$ .

*Method of Assay*—The following equations can be written for the oxidation of D-alanine by D-amino acid oxidase when coupled to the LDH system



The rate of oxidation of DPNH as written is dependent on the rate of

<sup>2</sup> This and all the following steps in the preparation of the apooxidase may be carried out equally well at room temperature or at  $4^{\circ}$ , the latter temperature insures the stability of the enzyme during preparation but prolongs the treatment necessary to separate the coenzyme from the protein.



pyruvate formation in Equation 1 when an excess of LDH is present. Likewise, pyruvate formation is dependent on the concentration of FAD in the presence of excess alanine. Therefore, under these conditions the rate of the over-all reaction is related to the degree of saturation of the enzyme with its coenzyme. As shown in Equation 1,  $H_2O_2$  is a product of the D-amino oxidase reaction, this must be destroyed, as it can oxidize pyruvate to acetate and  $CO_2$ . Accordingly, even though the enzyme pre-

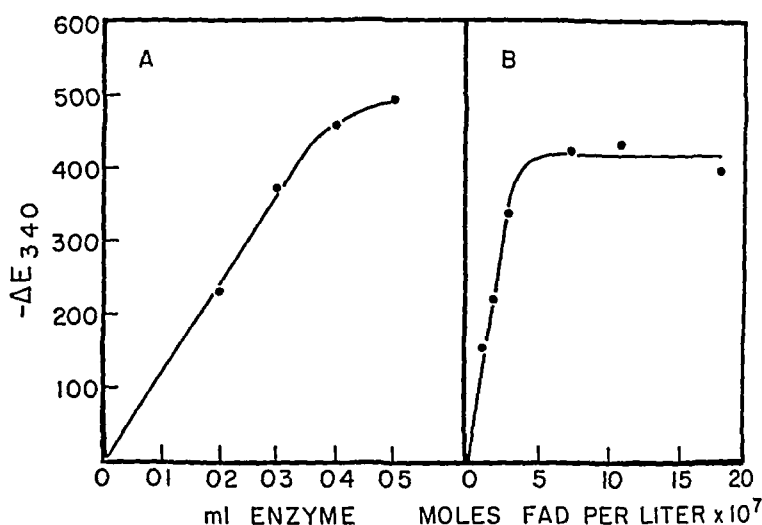


FIG 1 Concentration curves obtained by the D-amino acid oxidase system. The reaction mixtures contained 100  $\mu$ moles of phosphate buffer, pH 7.5, 0.34  $\mu$ mole of DPNH, 150 units of catalase, 0.1 mg of LDH protein, and 112  $\mu$ moles of DL alanine in a total volume of 3.0 ml. Fig 1, A, the reaction mixtures contained a final concentration of  $5.0 \times 10^{-6}$  M FAD and the enzyme concentrations as shown. B, the reaction mixtures contained 0.2 ml of apoenzyme and the FAD concentration shown. All reactions were started with alanine. Optical density changes are corrected for the slight change due to endogenous FAD bound to the apooxidase. A different apoenzyme preparation from that used in Fig 1, B was used to obtain the data shown in Fig 1, A.

pared as described above contained some catalase activity, catalase was always added in excess as part of the standard assay.

The standard assay adopted utilizes 150 units of crystalline catalase, 0.1 mg of crystallized protein of skeletal muscle LDH,<sup>3</sup> 0.3 to 0.4  $\mu$ mole of DPNH, 112  $\mu$ moles of DL-alanine, 100  $\mu$ moles of phosphate buffer, pH 7.5, 0.2 ml of D-amino acid apooxidase (approximately 0.25 mg of protein) prepared as described above, and 0.15 to 1.00  $\gamma$  (approximately  $0.15 \times 10^{-3}$  to  $1.5 \times 10^{-3}$   $\mu$ mole) of FAD in a final volume of 3.0 ml.

<sup>3</sup> Although the crude D-amino acid oxidase contained a very active DPN linked lactic dehydrogenase, this was lost during the preparation of the apooxidase and must be added in the assay.

As can be seen in Fig 1, *A* and *B*, the rate of oxidation of DPNH is proportional to apoenzyme and coenzyme concentrations, respectively. In the absence of either LDH or FAD no appreciable activity is observed. The apparent  $K_m$  for FAD under these conditions was found to be approximately  $1.8 \times 10^{-7}$  mole per liter. This value, however, depends somewhat on the efficiency of removal of the prosthetic group from the enzyme protein. Values have been reported (8) which range from 1.3 to  $2.5 \times 10^{-7}$  mole per liter at pH 8.3.

*Determination of FAD*—By using the linear portion of the coenzyme saturation curve, as illustrated in Fig 1, *B*, it was possible to determine FAD levels between 0.15 and 0.90  $\gamma$  ( $0.15 \times 10^{-3}$  to  $0.85 \times 10^{-3}$   $\mu$ mole).

TABLE I  
FAD Content of Rat Organs Obtained by Various Methods

Organ	FAD, $\gamma$ per gm fresh tissue				
	Present work	Method 1*	Method 2†	Method 3‡	Method 4§
Liver	54.8	31.0	77.3	36.6	45
Kidney	30.0	24.0	61.0	34.2	20
Heart	18.9	18.2	64.5	19.7	60
Spleen	8.0	3.4		6.1	

\* Fluorometric determinations, Bessey *et al* (7). The average values are listed.

† Manometric determinations accomplished with the D-amino acid oxidase system, Ochoa and Rossiter (10). The mean values are listed.

‡ Fluorometric method of Yagi (11). The average values are listed.

§ Manometric determinations accomplished with the D-amino acid oxidase system, Warburg and Christian (1). The single values are listed.

in 3.0 ml. It was advisable to plot a new standard saturation curve with known FAD on the day of assay. Although not shown in Fig 1, other flavins tested did not interfere with the assay (9). FMN in concentrations 100-fold in excess could not substitute for FAD as coenzyme in this system. In addition, 3000-fold excess of the mononucleotide did not inhibit the D-amino acid oxidase in the presence of FAD when measured by the spectrophotometric procedure. The results of determinations of FAD content of animal tissues and of microorganisms are presented in Tables I and II. Values for FAD present in several organs of the rat, obtained by the method of assay described here, are given in Table I. These values are compared to those reported by previous authors who used other methods. It may be seen that in general the values obtained by the present method agree fairly well with those reported earlier.

Perfused rat liver was fractionated by differential centrifugation into the nuclear, mitochondrial, microsomal, and soluble subfractions. Spec-

trophotometric assays on each subfraction showed that more than 50 per cent of the total liver FAD was present in the mitochondria. This is of interest in view of the fact that the bulk of the pyridine nucleotides is in the soluble fraction of rat liver.<sup>1</sup>

TABLE II  
Comparative FAD Content of Various Microorganisms

Microorganism	Spectro photometric method	Fluorometric method		FAD, % of total flavin
	FAD per gm fresh weight	FAD per gm fresh weight	Total flavin, ribo flavin per gm fresh weight	
	(A)	(B)	(C)	(D)
	γ	γ	γ	
<i>C. kluyveri</i> ††	228.0	226.0	137.4	79.4
" " §	7.0	3.8	11.2	29.9
<i>Mycobacterium tuberculosis</i> 607†	12.3	35.5	33.0	17.8
" phlei 101†	12.6	49.6	44.5	13.6
<i>C. butylicum</i>	59.7	61.1	37.0	77.1
<i>L. delbrueckii</i> ¶	21.0	11.6	20.3	51.7
" arabinosus	72.6	62.6	36.1	96.3
<i>Pseudomonas fluorescens</i>	42.9			
<i>Saccharomyces cerevisiae</i>	12.9			
<i>Escherichia coli</i>	12.5			
<i>Neurospora crassa</i>	8.5			
<i>Achromobacter fischeri</i>	26.8			
<i>Xanthomonas pruni</i>	12.0			

\* Calculated from values given in Columns A and C as follows (Column A/ (2.09 × Column C)) × 100 = per cent FAD. (The factor 2.09 was obtained from the ratio of molecular weights of 786 and 376 for FAD and riboflavin, respectively.)

† The values were determined from dried cells and converted to fresh weight with the assumption that cells are 80 per cent water.

‡ The cells were grown in synthetic medium.

§ The cells were grown in undefined medium.

|| The cell extracts were made with 10 volumes of 5 per cent trichloroacetic acid.

¶ We wish to thank Dr. Fritz Lipmann for the culture of *L. delbrueckii*.

Presented in Table II are the values for FAD obtained from the analyses of a number of microorganisms. For purposes of comparison, several values obtained by the adapted fluorometric method are listed in Column B. It may be seen that in most cases there is fairly good agreement between the results obtained with either the spectrophotometric or the fluorometric method. The outstanding exceptions are those presented by the values obtained from the determinations on the mycobacterial cell.

This discrepancy may be attributed to dinucleotide type flavins, not active in the D-amino acid oxidase system, which, upon acid hydrolysis, yield FMN or free riboflavin. That this is not due to the presence of enzyme inhibitors was shown by experiments in which 90 to 100 per cent of added FAD was recovered. The apparent inconsistency of results obtained with *Lactobacillus delbrueckii* and with *Clostridium kluyveri* grown in an undefined medium may be due to the relative insensitivity of the adapted fluorometric procedure to very low levels of FAD.

Total flavin, determined by fluorescence after acid hydrolysis, is listed in Column C. From these values and those given in Column A, the FAD content, expressed as per cent of total flavin, may be calculated. In the case of *Clostridium butylicum*, *Lactobacillus arabinosus*, and *C. kluyveri* grown in synthetic medium, most of the cell flavin may be accounted for as FAD. The species of *Mycobacteria*, however, show a low value of FAD as per cent of total flavin.

It is of interest that the levels of FAD and total flavin in *C. kluyveri* appear to be controlled to extremes by differences in the culture medium. 137.4  $\gamma$  of flavin per gm (79.4 per cent FAD) were obtained from cells grown in synthetic medium, while only 11.2  $\gamma$  of flavin per gm (29.9 per cent FAD) were obtained from cells grown in undefined medium. This effect of the medium is at present under investigation.

FAD content of a few higher plants has also been measured by this procedure. The method is also applicable to the determination of FAD in purified enzymes.<sup>4</sup>

*Detection of D-Amino Acid Oxidase*—In addition to its use for the determination of the coenzyme, the method has been used for the rapid detection of the D-amino acid oxidase itself in the presence of excess FAD and substrate. In testing for the oxidase in crude homogenates, potassium cyanide at  $1.0 \times 10^{-3}$  M was added to inhibit the catalysis of DPNH oxidation by the homogenates. By using whole homogenates of rat kidney and liver it was found, as previously reported, that rat kidney is a much better source of the enzyme than is rat liver. The presence of D-amino acid oxidase in whole rat heart homogenate could not be readily detected, due to the activity of a potent, cyanide-insensitive DPNH oxidase.

#### DISCUSSION

A spectrophotometric method has been described for the rapid determination of microquantities of FAD. The presence of other flavins greatly in excess of required FAD does not interfere with this procedure. Con-

<sup>4</sup> By using the spectrophotometric method, it was possible to establish a new, triphosphopyridine nucleotide (TPN)-specific diaphorase from spinach leaf as an FAD enzyme (Avron, M., and Jagendorf, A. T., to be published).

centiations of FMN far in excess of those normally found in tissues have no effect on the described assay system. Recovery experiments on microorganisms and animal tissue extracts indicate that the method is applicable to within 90 to 100 per cent of added FAD.

In general there is fairly good agreement of values of FAD obtained with either the spectrophotometric method described here or the fluorometric method adapted from Bessey *et al* (7). One exception, in the case of *Mycobacteria* (Table II), emphasizes the specificity of the former method for FAD and indicates that the fluorometric procedure may not always be an accurate index of FAD concentration. These results also suggest the presence of naturally occurring flavin dinucleotide type compounds which are not identical with FAD. Such compounds are not without precedent, as indicated by the reports of Whitby (12), Huennekens *et al* (13), and Singer, Kearney, and Massey (14). Deamino FAD and other analogues of FAD do not react in the D-amino acid oxidase system (9), however, these compounds would act like FAD under the conditions of the fluorometric assay.

Data are given in this paper which illustrate a number of general applications for the spectrophotometric method. Besides providing a rapid method for assaying FAD, it can be used to detect D-amino acid oxidase in tissues. At present the method is being utilized to measure the synthesis of FAD in liver subfractions and to determine mechanisms involved in flavoprotein action.

#### SUMMARY

1 A rapid, specific spectrophotometric method has been developed for the quantitative determination of flavin adenine dinucleotide (FAD). The technique involves coupling the D-amino acid apooxidase to the lactate dehydrogenase system in the presence of catalase. The method may be used to determine as little as 0.15  $\gamma$  of FAD accurately and reproducibly.

2 FAD concentrations in animal tissue extracts have been determined by this method and compared to those available in the earlier literature. Determinations on subcellular fractions of perfused rat liver show the mitochondria to have the highest content of this dinucleotide.

3 By using this method, a survey of FAD levels of a number of microorganisms has also been carried out, and values obtained have been compared, in some cases, to those obtained with a fluorometric method. Differences between the two methods were observed in the case of species of *Mycobacteria*, which may suggest the presence in these cells of considerable amounts of riboflavin bound in complex structures not identical with FAD or FMN.

4 The procedure described has also been used for the detection of D-amino acid oxidase in tissues

*Addendum*—Walaas and Walaas (15) have reported recently that partial structural analogues of FAD as FMN and AMP inhibit the D-amino acid oxidase system as measured manometrically. It should be noted that we have not seen any inhibition of the oxidase system as measured spectrophotometrically with concentrations of mononucleotides at  $10^{-3}$  M and FAD at  $2 \times 10^{-7}$  M

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# LARGE SCALE SYNTHESIS AND PURIFICATION OF FLAVIN ADENINE DINUCLEOTIDE\*

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Flavin adenine dinucleotide (FAD) of natural origin was first prepared and characterized by Warburg and Christian (1, 2). Recently, methods for the preparation of FAD have been modified by the application of more modern techniques. Large scale paper chromatography has been used by Huennekens and associates (3, 4), column electrophoresis by Siliprandi and Bianchi (5), and column chromatography on powdered cellulose by Whitby (6, 7). These procedures resulted in the isolation of at least 90 per cent pure FAD.

An ingenious first chemical synthesis of the dinucleotide was achieved several years ago by Christie, Kenner, and Todd (8) by the condensation of salts of riboflavin-5'-phosphate with 2',3'-O-isopropylidene adenosine-5'-benzyl phosphorochloridate followed by the removal of protective groups.

Shuster, Kaplan, and Stolzenbach (9) have reported a preliminary attempt at synthesis of the dinucleotide with the trifluoroacetic acid anhydride (TFAA)-condensing agent of Bourne *et al* (10), this yielded a mixture that was coenzymatically active in the D-amino acid oxidase system. This paper presents a detailed extension of this latter synthesis of FAD by the direct condensation of flavin mononucleotide (FMN) and 5'-adenylic acid (AMP). Subsequent large scale isolation and purification by chromatography according to the method of Whitby (7) are described.

While this paper was in preparation, Huennekens and Kilgour (4) reported another direct synthesis of FAD, with the use of the carbodiimide type catalyst recommended by Khorana (11), and isolation of the dinucleotide by paper chromatography.

## Materials and Methods

FAD of 53.5 per cent purity was obtained from the Sigma Chemical Company, and synthetic FMN was a gift from the same company. AMP

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(My-B-Den) was purchased from the Ernst Bischoff Company, Inc., and trifluoroacetic acid anhydride was supplied by the Columbia Organic Chemicals Company, Columbia, South Carolina. Whatman ashless powdered cellulose, standard grade, and all organic solvents, reagent grade, were obtained through the Fisher Scientific Company.

Synthetic FAD was isolated and purified by column chromatography on powdered cellulose with the solvent system of Whitby (7) *n*-butanol-*n*-propanol-water (2:2:1 volumes, ratio). The biological activity of products was determined with the D-amino acid oxidase system coupled lactic dehydrogenase, as described in the preceding paper (12).

Light absorption, measured with the Beckman model DU spectrophotometer, as well as paper chromatography and constitutive analysis, were used to characterize further the dinucleotide and to determine the purity of the final product.

Adenine, as adenosine, was determined by the method outlined by Kaplan (13) or that of Kalckar (14), by using both crude snake venom pyrophosphatase and intestinal adenosine deaminase; the deamination was followed by measuring the increase at 240  $m\mu$  with the aid of the Beckman photomultiplier attachment, model No. 4300. Crude venom of the rattlesnake (*Crotalus adamanteus*) was obtained from Ross Allen's Reptile Institute as a lyophilized powder, and crude intestinal deaminase was purchased from the Armour Company. Ribose was determined by the orcinol method of Mejbaum as described by Taylor *et al.* (15), and phosphate, as inorganic phosphate, by the method of Fiske and Subbarow (16).

### Results

*Synthesis of FAD*—10.3 gm of FMN (monosodium salt, dihydrate) and 3.5 gm of AMP were thoroughly mixed in a dry mortar, and 100 ml of TFAA were added<sup>1</sup> in small portions with continuous stirring and grinding.

After all the anhydride was added and the solids were nearly completely dissolved, the mortar containing the dark amber-colored mixture was placed in an empty vacuum desiccator and closed from the air. The desiccator was then allowed to stand at room temperature in a dark cupboard for about 16 hours. At the end of this period of time it was connected to a vacuum, and excess TFAA was removed until either a black, thick, gummy residue or solid glass remained. This residue was triturated with 180 ml of cold, anhydrous ether. The bright yellow-orange precipitate formed was centrifuged, washed twice with 80 ml portions of ether, and then dried *in vacuo*.

<sup>1</sup> Caution. Due to the vesicatory properties of the vapor of this reagent, synthesis should be carried out in the hood, proper protection being provided for exposed body surfaces.

To the dried, yellow powder were added 150 ml of cold absolute ethanol, saturated with ammonia gas. The solid was well suspended and placed on ice for 30 minutes. The suspension was centrifuged and the precipitate again placed on ice, this time for 10 minutes, after resuspension in  $\text{NH}_3$ -saturated ethanol. The precipitate was spun off, washed twice with 80 ml portions of cold, absolute alcohol, and dried as before. The fine, granular, orange powder (designated AI) weighed 13 gm.

A sample of this alcohol-insoluble material was dried to constant weight *in vacuo* over  $\text{P}_2\text{O}_5$  at  $55^\circ$ . When assayed for coenzymatic activity in the D-amino acid oxidase system, with Sigma FAD as a standard,<sup>2</sup> the content of FAD was found to be 9.4 per cent.

*Isolation and Purification of FAD*—750 gm of Whatman cellulose powder were washed with 0.1 N HCl until washings were free of absorption at 260  $\text{m}\mu$ . The powder was then treated with a mixture of *n*-butanol-*n*-propanol-water (2:2:1) until the washings no longer absorbed appreciably in the region 375 to 450  $\text{m}\mu$ . The washed powder was then poured as a slurry into a chromatography column and allowed to settle and pack to give a cellulose bed 150 mm wide by 105 mm high.

75 gm of the synthetic reaction mixture (AI) were then placed on the column in three successive portions, each portion was dissolved in 125 ml of water, 250 ml of *n*-propanol, and about 100 ml of *n*-butanol, each solvent being added slowly in turn in the order listed. Upon the addition of excess butanol, and upon addition to the column, there occurred a slight precipitation, this caused no interference<sup>3</sup> as the material redissolved as the column was eluted.

The column was developed slowly in the dark by using the solvent system mentioned above. The elution of the flavins was followed with the Beckman model DU spectrophotometer by the absorption at 260 and 450  $\text{m}\mu$ , with the resulting elution pattern in Fig. 1. By taking advantage of extremely low mobility of the FAD band, the height of the column was greatly restricted. According to control separations,<sup>4</sup> the elution pattern expected from such a restriction would include a major peak containing a mixture of fast moving contaminants and a secondary peak containing the slow moving FAD. The absence of a discrete dinucleotide peak in this instance may be explained by an overloading of the physical capacity of the column.

<sup>2</sup> In all cases in which commercial FAD was used as standard, correct concentration was determined from the per cent purity as indicated by the Sigma Chemical Company and the dry weight after drying *in vacuo* over  $\text{P}_2\text{O}_5$  at  $55^\circ$ .

<sup>3</sup> It may be necessary to line the top of the column with a circle of filter paper to insure homogeneous distribution of flavins without disruption of the cellulose surface.

<sup>4</sup> Numerous separations on a small scale with the use of cellulose columns not restricted in height showed definite partitioning of five to six flavin components, the first of which was FAD.

When the elution of all flavins was complete, the total effluent was divided arbitrarily into five sections of equal volume, as indicated in Fig 1, and aliquots from each composite section were extracted into water and assayed with the use of the D-amino acid oxidase system described in the preceding paper (12). The final 36 liters of effluent which contained the coenzymatic activity were extracted with chloroform, 10 volumes of eluate being reduced to 1 volume by the addition of 5 volumes of  $\text{CHCl}_3$ . The combined aqueous layers were extracted several times more, then lyophilized to dryness in the dark. This yielded a fluffy, bright orange material weighing a total of 980 mg (designated as C). A sample of this material,

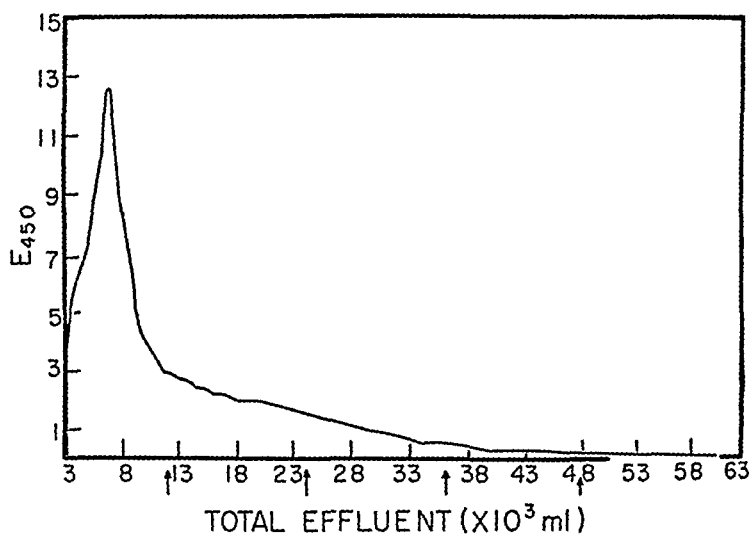


FIG 1 Elution of flavins from large preparative column. Aliquots of effluent were diluted with a mixture of *n*-butanol-*n*-propanol-water (2:2:1) and read against the same solvent as blank in the Beckman model DU spectrophotometer. Arrow heads indicate points of arbitrary section of the total effluent.

dried to constant weight over  $\text{P}_2\text{O}_5$  at  $55^\circ$ , showed a FAD content of 65.0 per cent when assayed by the D-amino acid oxidase system.

In an attempt to purify this material further, 200 mg were carried through the same procedure once again with a smaller column, 75 mm wide by 110 mm high. From this column, as shown in Fig 2, one major peak was obtained with a shoulder, both of which were coenzymatically active. These were separately extracted and lyophilized as before (designated C-II and C-III, respectively), a total of 113 mg was obtained, no effort being made at quantitative recovery.

*Characterization and Determination of Purity*—Samples of all isolated materials analyzed were dried as before over  $\text{P}_2\text{O}_5$  and dissolved in water. Table I shows the FAD content of several isolated fractions. Comparisons of ratios of optical densities at characteristic wave lengths are also given,

50  $\gamma$  samples dissolved in a total volume of 30 ml with 0.1 M phosphate buffer, pH 7.5, were used for the spectral studies. These results illustrate that extinction data alone are not suitable as criteria of purity of FAD.

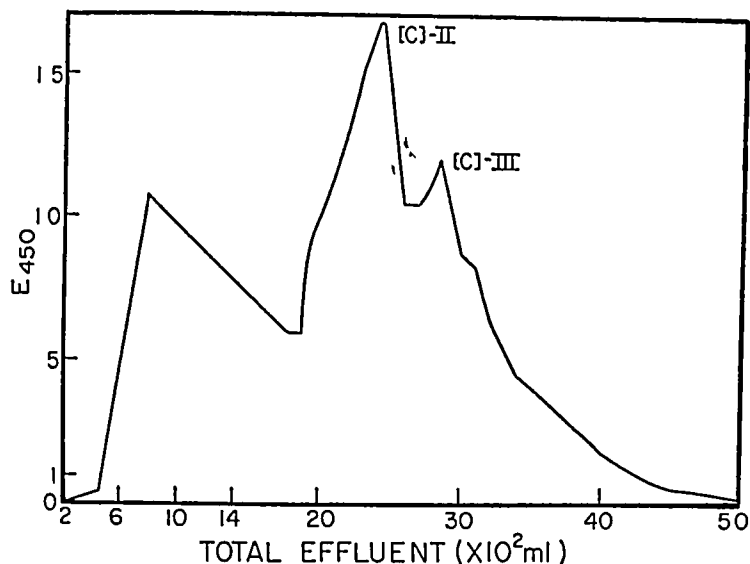


Fig. 2. Purification chromatography of isolated FAD. Aliquots of effluent were diluted with solvent mixture and read against the same solvent as blank in the spectrophotometer. Peak C-II was found to be 95 per cent pure FAD.

TABLE I  
*Relative Absorption Properties and Purity of Several Isolated Fractions*

Flavin*	$\frac{E_{260}}{E_{450}}$	$\frac{E_{375}}{E_{450}}$	Purity, per cent	
			(a)	(b)
Preparation AI	2.72	0.86		9.4
" C	3.12	0.85	98.4	66.0
" C-II	3.30	0.83	95.1	95.3
" C-III	3.00	0.86	97.5	39.4

Theoretical ratios of light absorption,  $\frac{260}{450}$  and  $\frac{375}{450}$ , have been reported (7) at 3.27 and 0.82, respectively. Per cent purity is calculated on the basis (a) of light absorption, assuming  $E_{450} = 1.13 \times 10^7$  sq. cm. per mole (2, 7) and (b) of coenzymatic activity in the D-amino acid oxidase system by using Sigma FAD as standard, details of which are given elsewhere (12).

\* For explanation of symbols, see the text.

The absorption spectrum of preparation C-II, assumed to be 95 per cent pure FAD, is shown in Fig. 3. Ultimate constitutive analysis of this material showed a flavin-phosphate-ribose-adenine ratio of 1.0:2.08:1.05.

1 05 The results shown in Table II indicate that C-II is almost pure FAD

Comparative experiments indicate that this purified product has co-enzymatic properties ascribed to FAD of natural origin. It was found that preparation C-II could activate the *Neurospora* nitrate reductase

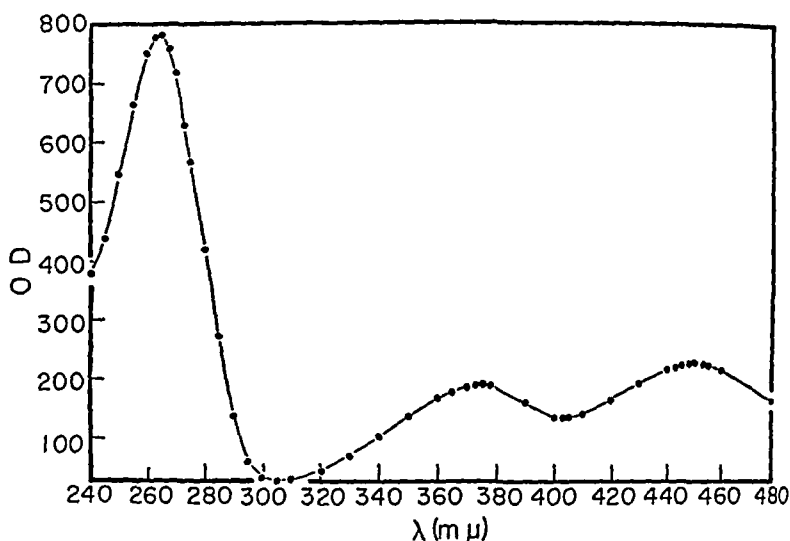


FIG 3 Absorption spectrum of fraction C-II. Spectrum obtained with 50 γ of C-II dissolved in a total volume of 3.0 ml. with 0.1 M phosphate buffer, pH 7.5

TABLE II  
Constitutive Analysis of Various Flavin Fractions

Flavin*	Total P, per cent	Ribose Flavin†	Adenine Flavin†
Preparation C	8.02	0.90	0.76
" C-II	8.28	1.05	1.05
" C-III	8.98	0.65	0.50

\* For explanation of the symbols, see the text

† Flavin based on extinction at 450 mμ

system which is known to utilize FAD for full activity (17), furthermore, it inhibited the *Achromobacter fischeri* luminescence system which is also inhibited by natural FAD (18)

*Synthesis of Analogues of FAD*—It was the ultimate purpose of this study to devise a method for the preparation of analogues of FAD which might aid in the study of the mechanism of action of flavin enzymes. Accordingly, a number of non-physiological dinucleotides of riboflavin have been prepared by varying purine-pyrimidine mononucleotides to be con

densed with FMN in the presence of TFAA, analogues containing guanine, cytosine, and uracil were prepared. Another analogue was prepared by the similar coupling of FMN with nicotinamide mononucleotide (NMN). Finally the deamino FAD was prepared by deamination with  $\text{HNO}_2$  according to the method of Kaplan, Colowick, and Ciotti (19) for the preparation of deamino DPN.

The precipitated crude reaction mixture<sup>5</sup> in each case was assayed directly in the D-amino acid oxidase system. In every instance the compounds formed were found to be biologically inactive as either activators or inhibitors in this system. It is evident that the adenine nucleus is essential for coenzymatic function and that its replacement results in a loss of activity.

### DISCUSSION

FAD has always been relatively unavailable in purities and amounts necessary for detailed chemical and enzymatic studies. It was desirable that some method be perfected for a large scale preparation of this coenzyme in high purity. Trifluoroacetic acid anhydride has been employed as a catalyst for the condensation of 5'-mononucleotides to dinucleoside pyrophosphates (20). A preliminary attempt by Shuster *et al* (9) to prepare the mixed dinucleotide, FAD, with the use of this reagent gave a crude reaction mixture which was able to reactivate the D-amino acid apooxidase. The work reported here is an extension of this preliminary synthesis and describes a simple, practical method suitable for a large scale isolation and purification of the coenzyme.

It is evident from the preparation of this naturally occurring dinucleotide that this method affords a means for the rapid synthesis of analogous compounds which may aid in the study of the mechanism of flavin participation in biochemical systems. Four analogues have been synthesized by varying 5'-mononucleotides in the reaction with FMN. A fifth analogue has also been prepared by chemical deamination of FAD with  $\text{HNO}_2$ . In every instance the analogue has proved to be inactive as coenzyme for the D-amino acid oxidase system. Thus it appears that the oxidase system is specific for the adenine moiety of the flavin dinucleotide. Analogues in which substituted flavin mononucleotides are being condensed with AMP are now in the process of preparation.

The authors wish to express their gratitude for the studies performed

<sup>5</sup> Analogues synthesized by the TFAA method are precipitated with ether as described above. The deamino FAD may be precipitated by either of two ways: (1) with barium acetate from solution neutralized with KOH, or (2) with 5 volumes of cold acetone from a solution made acid to Congo red with  $\text{HNO}_3$ .

by Mr Stephen C Kinsky and Mr Palmer Rogers with the *Neurospora* nitrate reductase and *A. fischeri* luciferase systems, respectively

#### SUMMARY

1 A synthesis of flavin adenine dinucleotide (FAD) is described in which riboflavin-5'-phosphate and 5'-adenylic acid are coupled directly in the presence of trifluoroacetic acid anhydride

2 The dinucleotide, active as coenzyme in the D-amino acid oxidase system, has been isolated and purified by column chromatographic method suitable for large scale preparations

3 The purified product has been characterized and estimated to be 99 per cent pure FAD

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# THE DIGESTION AND ABSORPTION OF ACETYLTRYPHTOPHAN

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In a recent paper (1) data were presented to show that acetyl-D-tryptophan was excreted in the feces, whereas acetyl-L-tryptophan was preferentially absorbed and utilized. Three possibilities were presented to explain the absorption of the L isomer: (a) It is deacetylated when passing through the gastrointestinal tract, (b) it is absorbed as acetyl-L-tryptophan and then deacetylated, and (c) both (a) and (b) may occur. This paper presents experimental data taken in an attempt to learn more about the mechanism of digestion and absorption of acetyltryptophan.

## Methods and Results

Acetyl-L-tryptophan was prepared as before (1). Acetyl-DL-tryptophan was synthesized by the acetylation of DL-tryptophan (2). In order to determine whether gut mucosa contains an enzyme which will deacetylate acetyltryptophan (1, 3), a section of fresh dog duodenum and jejunum was flushed with water and split longitudinally. All visible parasites were removed, and the mucosa was then separated as directed by Hawk, Oser, and Summerson (4).

5 ml. of the aqueous extract of the mucosa were incubated at 37°, pH 6.0 to 8.5, with 2 mg. of acetyl-L-tryptophan for 24 hours. Chromatographs of the incubation sample indicated that no hydrolysis had occurred. All chromatographs were resolved on Whatman No. 1 paper in a solvent system of methanol-butanol-benzene-H<sub>2</sub>O (2:1:1:1) (5) and developed with *p*-dimethylaminobenzaldehyde. In this system L- and D-tryptophan give slightly different *R<sub>F</sub>* values, whereas DL-tryptophan yields an elongated spot. From this evidence it was concluded that the digestive enzymes secreted by the gut mucosa do not hydrolyze acetyltryptophan.

The structure of acetyltryptophan suggests that carboxypeptidase would attack the molecule. Using acetyl-DL-tryptophan as the substrate, Putnam and Neurath (6) showed that 17.9 per cent hydrolysis occurred within 105 minutes of incubation with carboxypeptidase crystallized either five or seven times. Gilbert *et al.* (7) reported a rate of hydrolysis of only 6  $\mu$ moles for acetyl-DL-tryptophan per hour per mg. of enzyme N.

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Carboxypeptidase, crystallized three times, obtained from the Worthington Biochemical Corporation, was diluted to contain 0.083 mg of N per ml with 0.02 M phosphate buffer, pH 7.7, containing 0.46 mg per cent of  $MgCl_2$ . Aliquots of this solution were incubated in a Dubnoff metabolic shaker at  $37^\circ$  with an equal volume of a 0.05 mM solution of acetyl-D or acetyl-L-tryptophan in buffer or a 0.10 mM solution of acetyl-DL-tryptophan in buffer. At 0, 1, 2, 4, and 24 hours, aliquots of the incubation mixture

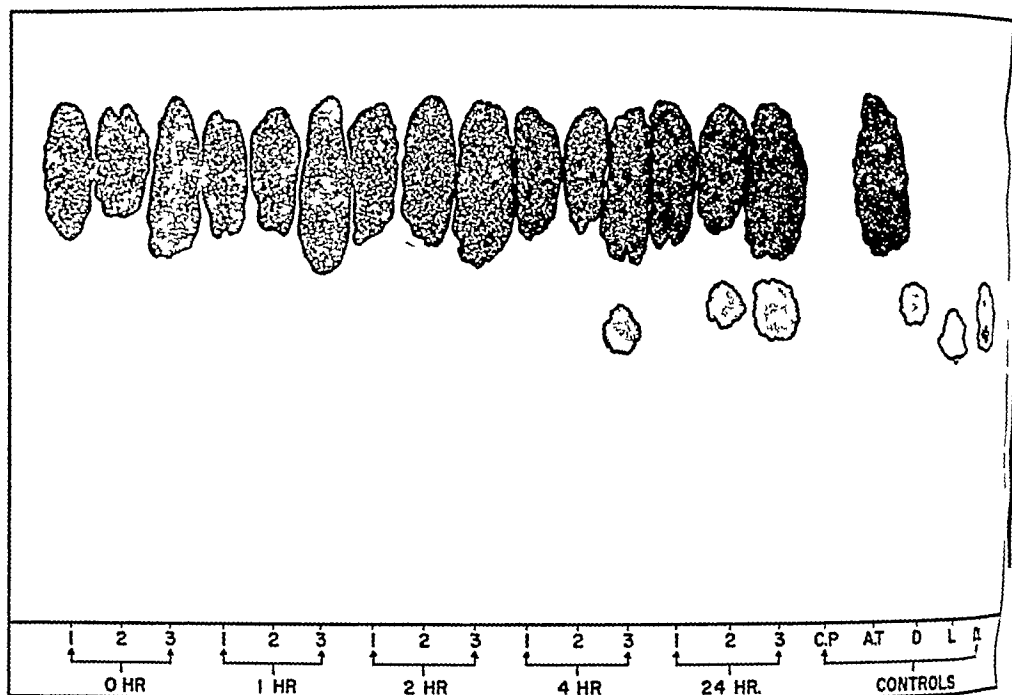


FIG 1 Hydrolysis of acetyltryptophan by carboxypeptidase. Chromatograph resolved in methanol-butanol-benzene- $H_2O$  (2:1:1:1) and developed with 2 per cent *p*-dimethylaminobenzaldehyde. Spot 1, acetyl-D-tryptophan with carboxypeptidase, Spot 2, acetyl-L-tryptophan with carboxypeptidase, Spot 3, acetyl-DL-tryptophan with carboxypeptidase.

were withdrawn. The enzymatic action was stopped by boiling for 5 minutes, and the coagulated protein was removed by centrifugation. The filtrate was chromatographed with 20  $\mu$ l applications. Incubation samples containing acetyl-D-tryptophan as substrate did not yield a spot for D-tryptophan, indicating that no hydrolysis had occurred. Samples with acetyl-L- or acetyl-DL-tryptophan as substrate yielded a spot for L-tryptophan after 1 hour incubation. At subsequent time intervals the spot became more pronounced (Fig 1).

Quantitative results from microbiological assays supported the chromatographic evidence that hydrolysis of the acetyl-L and acetyl-DL analogue

had occurred. Essentially, the assay method for determining L-tryptophan with *Lactobacillus plantarum*, ATCC 8014, was similar to that described by other workers (8, 9). Both the basal medium described by Stokes *et al* (10) and the tryptophan assay medium from the Difco Laboratories were found satisfactory. Incubation was carried out at 31° for 18 hours, and the growth response of the test organism was measured turbidimetrically in a Klett-Summerson colorimeter at 660 m $\mu$ . In preliminary investigations it was found that *L. plantarum* did not utilize the acetylated isomers of tryptophan. Quantitative measurements of L-tryptophan could be obtained in the presence of the acetylated analogue at the concentration

TABLE I  
*Hydrolysis of Acetyltryptophan by Carboxypeptidase\**

Substrate in assay aliquot		Time of incubation at 37.5°							
		1 hr		2 hrs		4 hrs		24 hrs	
		L-Tryp- tophan	Hydroly- sis	L-Tryp- tophan	Hydroly- sis	L-Tryp- tophan	Hydroly- sis	L-Tryp- tophan	Hydroly- sis
		$\gamma$	per cent	$\gamma$	per cent	$\gamma$	per cent	$\gamma$	per cent
Acetyl L-tryptophan	6.15	0.20	3.25	0.25	4.07	0.43	6.99	1.40	22.76
	4.31	0	0	0.17	3.95	0.35	8.12	1.00	23.20
	1.85	0	0	0	0	0.17	9.19	0.43	23.24
Acetyl DL-tryptophan	6.15	0.40	6.50	0.50	8.13	1.00	16.26	4.30	69.92
	4.31	0.20	4.64	0.40	9.28	0.75	17.40	3.00	69.61
	1.85	0	0	0.20	10.81	0.30	16.22	1.22	65.95

\* Measured by microbiological assay with *L. plantarum* 8014 and calculated from a standard curve.

ratios expected in the assay of the carboxypeptidase-acetyltryptophan incubation mixture.

The incubation samples for assay were diluted so that the amount of L-tryptophan formed on hydrolysis would fall within the effective measuring range on the standard curve. Both chromatography and microbiological assay demonstrated that acetyl-DL-tryptophan was hydrolyzed to a greater extent than was acetyl-L-tryptophan (Table I).

The slow rate at which acetyltryptophan was hydrolyzed *in vitro* indicated that carboxypeptidase is not the only mechanism of deacetylation and subsequent utilization. In order to determine whether acetyltryptophan is absorbed before deacetylation, fourteen rats, fasted 48 hours, were fed 1.0 mmole (246 mg) of acetyl-L-tryptophan as the sodium salt by stomach tube. At hourly intervals two rats were sacrificed and blood was withdrawn from the vena cava. The blood was deproteinized with phosphotungstic acid, and 50  $\mu$ l of the filtrate were chromatographed. At

each time interval acetyltryptophan was detected. Microbiological assay of the blood filtrate showed a rise in the L-tryptophan content at 6 hours. The filtrate from control rats fed an equivalent amount of L-tryptophan showed a peak at 3 hours (Fig. 2). Quantitative data on the differences of absorption of the free and acetylated tryptophan isomers will be presented in a succeeding paper.

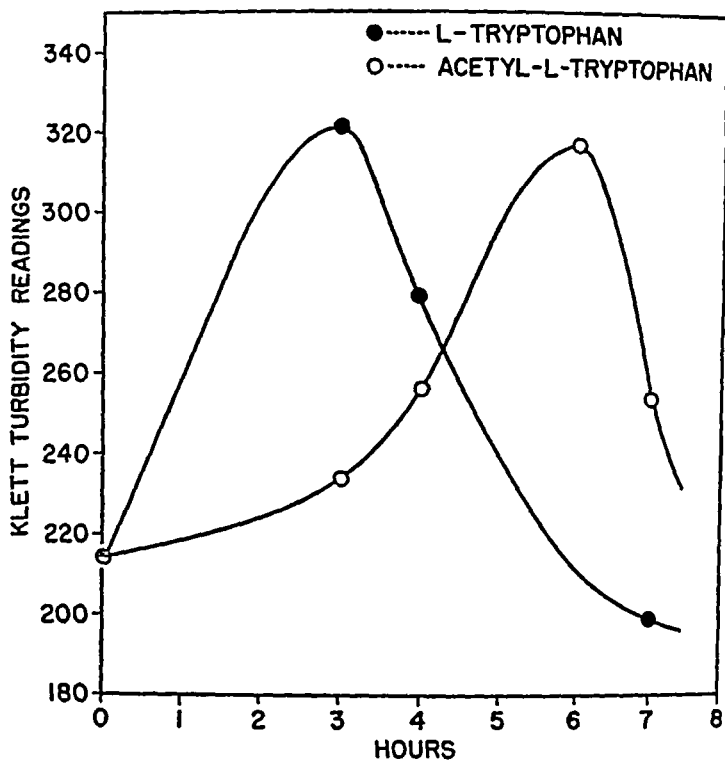


Fig. 2 L-Tryptophan content in blood of rats fed equivalent amounts of L-tryptophan and acetyl-L-tryptophan. Measured by microbiological assay with *L. plantarum* 8014. Growth response of the test organism measured turbidimetrically on a Klett-Summerson colorimeter at 660  $m\mu$ .

#### DISCUSSION

Although carboxypeptidase does hydrolyze acetyl-L-tryptophan, the rate appears to be too slow to be of importance. The early appearance of acetyl-L-tryptophan in the blood of rats indicates that some of the ingested acetyltryptophan is absorbed before deacetylation can occur in the gut. It is probable that acylase, a deacetylating enzyme found in the liver and kidney of several species of mammals (1, 4), is responsible for the deacetylation and subsequent utilization of acetyl-L-tryptophan. The rise in the L-tryptophan content of the blood 6 hours after ingestion of the acetylated isomer may be the result of either deacetylation of the absorbed acetyl-

tryptophan by acylase or the absorption of the free tryptophan formed by the action of carboxypeptidase on the acetyltryptophan remaining in the gut

The effect of the acetyl-D-tryptophan in a racemic mixture on an enzyme such as carboxypeptidase is difficult to explain. The possibility of contamination of the acetyl-DL-tryptophan was eliminated when chromatographs and a negative ninhydrin reaction showed the complete absence of free tryptophan. Consistent results were obtained with new batches of carboxypeptidase and acetyltryptophan and with a mixture of equal portions of acetyl-D- and acetyl-L-tryptophan. The same effect can be demonstrated *in vivo*. When rats fed acetyl-DL-tryptophan were subjected to the Cori technique (11) for measuring absorption and the gut filtrate was assayed for free L-tryptophan, a sharp increase in bacterial growth occurred at the 6 hour interval. Gut filtrates of rats fed acetyl-L-tryptophan showed no increase in L-tryptophan at any time interval. The increased hydrolysis of the racemic mixture shows that the D isomer does not compete for the favored position on the enzyme molecule. Further work is in progress to evaluate the effect of the acetylated D isomer in the racemic mixture on the action of carboxypeptidase.

#### SUMMARY

Carboxypeptidase hydrolyzed acetyl-DL-tryptophan at a greater rate than it did acetyl-L-tryptophan even though it is specific for the L isomer. The rate of hydrolysis was considered too slow to be of importance in the absorption and utilization of acetyl-L-tryptophan. Acetyl-L-tryptophan was found in rat blood shortly after its ingestion. It was concluded that the major portion of acetyl-L-tryptophan was absorbed before deacetylation.

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# ABSORPTION STUDIES ON TRYPTOPHAN AND ACETYLTRYPTOPHAN

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Many investigations have demonstrated that amino acids are absorbed by enzymatic processes (1-3). Recent investigations on the fate of ingested acetyl-L-tryptophan (4) showed that the rate of absorption of this isomer is much slower than that reported previously for an equivalent amount of free L-tryptophan (5). du Vigneaud *et al.* (6) found, however, that the acetylated analogue is utilized equally as well as the free form for growth in rats, and Baldwin and Beig (7) reported that acetyl-L-tryptophan will maintain nitrogen balance in humans. Therefore, the difference in the rate of absorption suggested that a comparison of the absorption of the various isomers of free and acetyltryptophan might help clarify the action of the enzymes concerned in this process. This paper presents experimental data on the absorption of D-, L-, DL-, acetyl-D-, acetyl-L-, or acetyl-DL-tryptophan from the gastrointestinal tract of rats.

## *Methods and Results*

The free amino acids used in this study were obtained from the Nutritional Biochemicals Corporation. The acetylated isomers were prepared as before (8, 9). In each experiment twenty-one rats of the Sprague-Dawley strain, weighing approximately 150 gm, were fasted for 48 hours. They were then divided into seven groups of three rats each. Five of the groups were fed 1 mmole of the test compound as its sodium salt by stomach tube. Preliminary studies had shown that some absorption occurs immediately after feeding. Therefore, the sixth group was given an intraperitoneal injection of Nembutal, fed the amino acid solution, and sacrificed within 10 minutes to obtain 0 hour results. The seventh group was used as unfed controls.

At 2 hour intervals up to 10 hours, a group of rats was anesthetized and the abdominal cavity was opened. Blood was withdrawn from the vena cava in a heparinized syringe and deproteinized with phosphotungstic acid. The gastrointestinal tract was tied at each end to prevent loss of contents and then removed. The gut was freed of excess tissue, cut in  $\frac{1}{4}$  inch sec-

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# ABSORPTION OF TRYPTOPHAN ISOMERS

, and suspended in 500 ml of  $H_2O$ . The extract was then filtered through gauze to obtain a relatively clear solution.

The blood filtrates were assayed with *Lactobacillus plantarum* (10, 11) for free tryptophan. A sharp rise in the free tryptophan content in the blood of rats fed L- or DL-tryptophan occurred at the 2 hour interval. In the rats fed acetyl-L- or acetyl-DL-tryptophan, the rise occurred at the 6

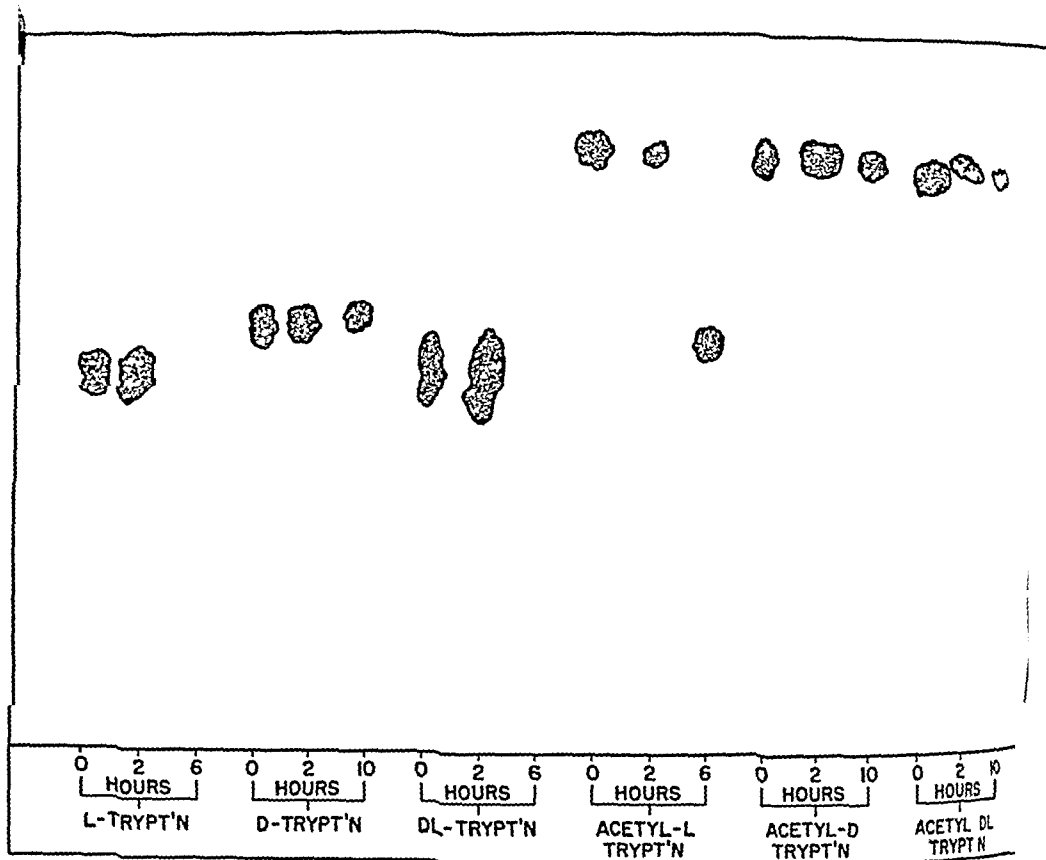


FIG 1 Tryptophan and acetyltryptophan in the blood filtrate from rats fed 1 mmole of the isomer

hour interval. Blood and gut filtrates were chromatographed as previously described (4). Figs 1 and 2 represent composite drawings of all the chromatographs.

Tryptophan or acetyltryptophan in both blood and gut filtrates was determined by using the Shaw-McFarlane chemical method (12). With the 48 hour fasting period, only slight variations in results were found among individual rats within a group. The values reported are group averages corrected by subtracting the average value obtained for the control group. Table I summarizes the results of the chemical analysis and



FIG 2 Disappearance of tryptophan and acetyltryptophan from the gastrointestinal tract of rats fed 1 mmole of the isomer

TABLE I  
Absorption of Tryptophan and Acetyltryptophan  
from Gastrointestinal Tract of Rats\*

Hrs	L-Tryptophan			D-Tryptophan			DL-Tryptophan			Acetyl L tryptophan			Acetyl-D-tryptophan			Acetyl DL tryptophan		
	In gut	Absorbed	In blood	In gut	Absorbed	In blood	In gut	Absorbed	In blood	In gut	Absorbed	In blood	In gut	Absorbed	In blood	In gut	Absorbed	In blood
	mg	per cent	mg per cent	mg	per cent	mg per cent	mg	per cent	mg per cent	mg	per cent	mg per cent	mg	per cent	mg per cent	mg	per cent	mg per cent
0	140	31 4	12	130	36 3	18	150	26 5	13	225	8 5	17	190	22 8	7	200	18 7	3
2	29 5	85 5	55	115	43 6	18	22 5	89 5	25	150	39 0	7 4	183	25 6	10	167	32 1	4 5
4	0	100	17 5	75	63 2	19	11 2	94 5	6	156	36 6	6 0	188	23 6	10	170	30 9	0
6	0	0	0	75	63 2	16	7 5	96 3	4 5	145	41 1	3 0	190	22 8	15	160	35 0	0
8	0	0	0	65	68 1	10	7 3	96 4	0	125	49 2	3 0	187	24 0	15	115	53 3	0
10	0	0	0	50	75 5	10	7 2	96 5	0	112	54 5	3 0	205	16 7	15	122	50 4	0

For L-, D-, and DL-tryptophan, 204 mg dose, for acetyl-L-, acetyl-D-, and acetyl-DL tryptophan, 246 mg dose

\* Measured by the Shaw-McFarlane chemical procedure



clearly shows that the rates at which tryptophan and acetyltryptophan are absorbed and utilized vary widely

### DISCUSSION

The results of chemical, microbiological, and chromatographic analyses agree with those of earlier reports that L-tryptophan is rapidly absorbed from the gastrointestinal tract and disappears quickly from the blood stream (5). Although 204 mg is a large dose for a rat in terms of daily requirements, the animals were capable of absorbing all of the L isomer within 4 hours after ingestion. The concentration curve of L-tryptophan in the blood shows that the isomer disappears from circulation almost as rapidly as it is absorbed.

Although the initial absorption of D-tryptophan compares with that of L-tryptophan, the absorption rate is reduced, and, after 10 hours, only 70 per cent of the ingested compound is absorbed. The slow rate of absorption of the isomer may aid in its subsequent utilization. Blood levels indicate that D-tryptophan is present even 10 hours after ingestion. Although the D-tryptophan is metabolized more slowly than the L isomer, Oesterling and Rose (13) found that it can be satisfactorily utilized by rats.

Results obtained from rats fed DL-tryptophan indicate that the L moiety of the racemic mixture is preferentially absorbed. A higher percentage of the D isomer in the racemic mixture is absorbed than might be expected from results obtained on D-tryptophan alone. It is possible that the enzyme system responsible for the absorption of D-tryptophan was overwhelmed by the 204 mg dose, but, with a reduced amount of D isomer available in the racemic mixture (102 mg), greater absorption could occur. However, the sharp decrease in the rate of absorption of the DL-tryptophan observed at the 4 hour interval suggests that the L isomer has been completely absorbed by this time and that only the D isomer remains. This is then absorbed at the slow rate observed with D-tryptophan alone. Therefore, it seems that the presence of L-tryptophan in a racemic mixture enhances the absorption of the D isomer. The influence of the L isomer on the utilization of the D enantiomorph of tryptophan in growth studies with mice has been reported (14).

The absorption rate of acetyl-L-tryptophan is similar to that of D-tryptophan. At the end of 10 hours, only 55 per cent of the ingested compound had been absorbed, yet the acetylated isomer has been found to be equivalent to the free form in supporting growth in rats. The level of acetyl-L-tryptophan in the blood immediately after ingestion of the compound is comparable to the amount of D-tryptophan found in blood filtrates of rats fed the D isomer. The sharp decrease in this level within 2 hours suggests that the body can deacetylate and utilize acetyl-L-tryptophan faster than

it can be absorbed. The measurable amount of acetyl-L-tryptophan in the blood after 10 hours indicates that absorption continues at a slow rate even in the large intestine.

Langner and Volkmann (8) have shown that 75 per cent of ingested acetyl-D-tryptophan is not absorbed by the human subject. Similar results are obtained with rats. 10 hours after feeding acetyl-D-tryptophan to rats, only 16 to 25 per cent of the compound is absorbed. The high, constant level of acetyltryptophan in the blood suggests that the small amount of acetyl-D-tryptophan absorbed is not utilized and probably is eventually excreted in the urine.

The specificity of the enzymes concerned in absorption is clearly shown with acetyl-D-tryptophan. If a free amino group is not essential for absorption as demonstrated by acetyl-L-tryptophan, and if the stereoarrangement is not important as shown with D-tryptophan, then the effect of acetylation of D-tryptophan presents an interesting problem. It is possible that the acetyl group is large enough to produce a steric effect between the enzyme and substrate, thus preventing the formation of an enzyme-substrate complex.

The data obtained from analyses of the blood and gut filtrates of rats fed acetyl-DL-tryptophan demonstrate that the D enantiomorph does not inhibit the absorption of the L isomer by competing for a favored position on the enzyme molecule. Conversely, the presence of acetyl-L-tryptophan in the racemic mixture does not enhance the absorption of the acetyl-D isomer. The low blood level and rapid disappearance of acetyltryptophan from the blood of the rats fed the racemic mixture support the idea that very little acetyl-D isomer is absorbed. Thus, the increase in the total amount of acetyl-DL-tryptophan absorbed is mostly due to the absorption of only the L moiety. When the gut filtrates were extracted with ether to remove acetyltryptophan, the extracts were evaporated to dryness, and the residue was taken up in 0.01 N NaOH, a negative polarimeter reading resulted. Control animals gave a 0 reading. This substantiates the idea that a large part of the acetylated L isomer had been preferentially absorbed, leaving acetyl-D-tryptophan in the gut.

Up to the 8 hour interval the rates at which each of the isomers in the racemic mixture was absorbed corresponded to those found when each isomer was administered separately. At the 8 hour interval a greater amount of acetyl-DL-tryptophan had disappeared from the gut than could be accounted for on this basis, and the L-tryptophan level in both blood and gut filtrates increased at this time interval, as demonstrated by *L. plantarum*. In earlier work (4) it was found that acetyl-D-tryptophan was not hydrolyzed by carboxypeptidase. Deacetylation of acetyl-DL-tryptophan began after only 4 hours incubation with carboxypeptidase, and the

rate of hydrolysis increased on prolonged incubation. The above evidence suggests that the decrease in the amount of acetylated racemic mixture remaining in the gut can be attributed to the deacetylation of the acetyl-L isomer to form free L-tryptophan which is then rapidly absorbed. It is unlikely that the free form remains in the gut long enough to enhance the absorption of the acetylated D enantiomorph.

When the dosage of acetyl-DL-tryptophan was reduced to 0.5 mmole (123 mg), 67 per cent of the compound was absorbed. Apparently the enzymes concerned with the absorption of the acetylated analogues were easily overwhelmed by large doses of the compounds. Feeding the compounds with a complete diet or reducing the amounts administered during an experiment may alter considerably the rate of absorption and utilization of the individual isomers.

#### SUMMARY

Fasted rats were fed free or acetyltryptophan. The disappearance from the gastrointestinal tract and the concentrations in the blood were followed for 10 hours. Free L-tryptophan was absorbed and utilized most rapidly and acetyl-D-tryptophan the least rapidly. Differences among the various isomers are discussed.

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# THE ENZYMATIC TRANSFER OF HYDROGEN

## VI THE REACTION CATALYZED BY D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE\*

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The previous paper of this series (1) presented evidence that the reaction catalyzed by glucose dehydrogenase involves a direct transfer of hydrogen between glucose and the  $\beta$  side of the 4 ( $\gamma$  or para) position of the nicotinamide ring of DPN.<sup>1</sup> The present paper presents evidence that D-glyceraldehyde-3-phosphate dehydrogenase also causes direct transfer of hydrogen between the triose phosphate and DPN. Furthermore, the steric specificity of the reaction for DPN is  $\beta$ , just as it is for glucose dehydrogenase.

### Materials

Crystalline triosephosphate dehydrogenase was prepared from Fleischmann's bakers' yeast by the method of Kunitz and McDonald (2) as modified by Krebs *et al.* (3). The product was recrystallized twice, washed with 50 per cent  $(\text{NH}_4)_2\text{SO}_4$ , and stored under  $(\text{NH}_4)_2\text{SO}_4$  solution at 3–5°. Crystalline aldolase (4) and muscle triosephosphate dehydrogenase were prepared from rabbit muscle according to Cori *et al.* (5). Aldolase was recrystallized once and the dehydrogenase was recrystallized three times. Both preparations were stored under  $(\text{NH}_4)_2\text{SO}_4$  solution at 3–5°. A sample of muscle triosephosphate dehydrogenase obtained from the Nutritional Biochemicals Corporation was also employed. The triosephos-

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<sup>1</sup> DPN represents diphosphopyridine nucleotide,  $\text{DPN}^+$ , oxidized DPN, specifically, and  $\text{DPNH}$ , reduced DPN. The abbreviation DPND is used to represent reduced diphosphopyridine nucleotide containing D in the reduced or para position of the nicotinamide ring.  $\alpha$ -DPND is used to represent the stereoisomer of DPND obtained by reduction of  $\text{DPN}^+$  with ethanol-1,1- $d_2$  in the presence of yeast alcohol dehydrogenase.  $\beta$ -DPND then represents the other diastereoisomer of DPND, FDP, fructose 1,6 diphosphate, Tris, tris(hydroxymethyl)aminomethane.

phate dehydrogenase was assayed according to Krebs *et al* (3), and alcohol dehydrogenase was assayed according to Warburg and Christian (6) and Taylor *et al* (4)

For the experiments carried out in  $D_2O$ , a solution of FDP was prepared as follows 850 mg of the Ca salt of FDP (Schwarz) were dissolved in 2 ml of  $D_2O$  To this were added 0.2 ml of 6 N  $H_2SO_4$  made in  $D_2O$  and 370 mg of  $K_2C_2O_4 \cdot H_2O$  in 1.0 ml of  $D_2O$  The precipitate was centrifuged, resuspended in 1 ml of  $D_2O$ , and centrifuged again The first and second supernatant solutions were combined

A solution of DL-glyceraldehyde-3-phosphate was also specially prepared for the experiments carried out in  $D_2O$  70 mg of the Ca salt (Concord Laboratories) were suspended in 1.5 ml of  $D_2O$  and the pH was adjusted to 2 by the addition of 0.06 ml of 6 N  $H_2SO_4$  which had been prepared from concentrated  $H_2SO_4$  by dilution in  $D_2O$  To this was added 0.3 ml of a solution prepared by dissolving 184 mg of  $K_2C_2O_4 \cdot H_2O$  in 1 ml of  $D_2O$  The precipitate was centrifuged, resuspended in 1 ml of  $D_2O$ , and centrifuged again The supernatant solutions were combined and the pH was adjusted to 8 by the addition of 50 mg of  $K_2CO_3$

Two samples of nicotinamide-4-*d*-DPN<sup>+</sup> were used One of these was the same as that previously described (1) It had been prepared by formation and dissociation of the DPN<sup>+</sup>-cyanide complex in  $D_2O$  (7), without the addition of alkali, and contained 0.136 atom of D at the 4 position of the nicotinamide ring Another sample of nicotinamide-4-*d*-DPN<sup>+</sup> was prepared according to San Pietro (7) by adding 5 ml of 0.13 M KOH made with  $D_2O$  to a solution containing 1 gm of DPN<sup>+</sup> and 975 mg of KCN in 10 ml of  $D_2O$  The solution was allowed to stand at room temperature for 1 hour and 40 minutes The cyanide complex was dissociated by the addition of  $KH_2PO_4$  and the removal of HCN as described by San Pietro, and the DPN<sup>+</sup> was isolated by acetone precipitation and then purified by adsorption on and elution from a Dowex formate column (8) The DPN<sup>+</sup> was recovered from the eluate by lyophilization and repurified on a Dowex column prepared according to Hurlbert *et al* (9) The nicotinamide obtained from this DPN<sup>+</sup> by treatment of a sample with DPNase contained 0.477 atom of D per molecule All other materials have been previously described (1)

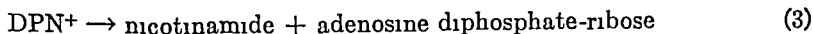
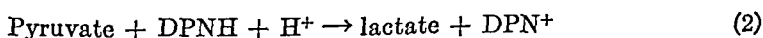
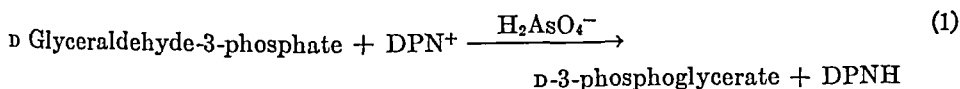
## RESULTS AND DISCUSSION

In a number of preliminary experiments, attempts were made to isolate DPNH by acetone precipitation after reduction of DPN<sup>+</sup> by the trioxophosphate dehydrogenase system in a medium of  $D_2O$  The results of these experiments indicated that the enzyme probably caused a direct transfer of hydrogen, since little non-exchangeable D was found in the DPNH, but

the yields of DPNH obtained were so small and the purity of the product was so low that this procedure was finally abandoned

In another set of experiments, DPN was reduced by the triose phosphate system in a medium of  $D_2O$ , and the reduced DPN was reoxidized by pyruvate in the presence of added lactic dehydrogenase, without prior removal of the  $D_2O$ . It was hoped that the enolization of pyruvate would not be sufficiently rapid to introduce much D into the lactate, but this hope was not realized. Analysis of lactate formed in this way gave 0.5 or more atom of D per molecule. The results of the final set of experiments showed that most of this D was probably introduced into lactate by prior enolization of the pyruvate. Apparently something in the triosephosphate dehydrogenase reaction mixture catalyzed this enolization, since pyruvate was previously shown not to enolize extensively in the lactic dehydrogenase reaction mixture (10).

The procedure finally adopted for studying the triosephosphate dehydrogenase reaction was similar to that previously employed in the study of hydrogen transfer and steric specificity with glucose dehydrogenase (1). Two types of experiments were performed, one designed to determine whether direct hydrogen transfer was involved and the other designed to determine the steric specificity of the reaction for DPN. In these two kinds of experiments, the deuterium label was employed differently, but the reaction sequence, shown in Equations 1 to 3, was the same



In one set of experiments,  $DPN^+$  was reduced by triose phosphate in the presence of enzyme and arsenate in a medium of  $D_2O$  (Equation 1). When the reaction was almost complete, the enzyme was heat-inactivated, and the  $D_2O$  was removed by lyophilization. The question was whether the DPNH had acquired non-exchangeable D from the medium during the reduction. This was determined as previously described (1) by reoxidizing the DPNH enzymatically with pyruvate (Equation 2) and hydrolyzing the  $DPN^+$  thus formed with DPNase (Equation 3). The lactate and the nicotinamide formed in these reactions were isolated and analyzed for D. The lactate will contain any D introduced into the  $\alpha$ -para position of the nicotinamide ring of DPNH, and the nicotinamide will contain any D introduced into the  $\beta$ -para position (or any other non-exchangeable position of the nicotinamide).

The results of a set of three experiments are given in Table I. Experiments 1 and 2 were both carried out with yeast triosephosphate dehydrogenase and were similar except that in Experiment 1 the D-glyceraldehyde-3-phosphate was generated from fructose-1,6-diphosphate by the action of

TABLE I  
*Demonstration of Direct Transfer of Hydrogen*

Experiment No	Dehydrogenase source	Lactate*			Nicotinamide		
		Atom per cent excess D found	Dilution factor	Atom D per molecule	Atom per cent excess D found	Dilution factor	Atom D per %
1	Yeast	0.058	19.1	0.13	0.028	36.6	0.06
2	"	0.021	29.0	0.07	0.009	120.0	0.06
3	Muscle	0.015	23.6	0.04	0.031	30.3	0.06

*Experiment 1*—To 5 ml of D<sub>2</sub>O were added, in the order given, 15 mg of KH AsO<sub>4</sub>, 20 mg of cysteine hydrochloride, 600 mg of Tris, 0.1 ml of concentrated HCl, 60 mg of DPN<sup>+</sup> of 85 per cent purity, and 0.8 ml of the solution of FDP in D<sub>2</sub>O (see under "Materials"). The volume was adjusted to 9.8 ml and the pH was 8.5. To this were added 0.1 ml of a suspension containing 8 mg of yeast triosephosphate dehydrogenase and 0.07 ml of a suspension containing 2.7 mg of aldolase. The reaction was followed by measuring DPNH spectrophotometrically at 340 mμ with suitable dilutions of small aliquots of the reaction mixture. At 25 minutes after enzyme addition, when no further reduction of DPN was occurring, the reaction flask was placed in a boiling water bath for 1.5 minutes to inactivate the enzymes and then cooled rapidly. The protein precipitate was removed by centrifugation. Enzymatic assays with acetaldehyde and alcohol dehydrogenase showed that 64 μmoles of DPNH were present. The solution was lyophilized to remove the D<sub>2</sub>O. The residue was dissolved in 3 ml of H<sub>2</sub>O and re-lyophilized, then the solids were taken up in 9.0 ml of H<sub>2</sub>O, and the pH was adjusted to 7.2 with 5 N HCl. Enzymatic assay showed that 57 μmoles of DPNH were present. The DPNH was reoxidized with an equivalent amount of sodium pyruvate in the presence of added lactic dehydrogenase, the enzyme was heat-inactivated, 100 mg of lithium L-lactate were added, and the pH was adjusted to 2 with N H<sub>2</sub>SO<sub>4</sub>. The lactic acid was extracted with ether and converted to phenacyl lactate which was analyzed for D, all as previously described (1). The aqueous residue from the ether extraction was adjusted to pH 8 with 1 N NaOH, and the DPN<sup>+</sup> was separated by adsorption on and elution from a Dowex formate column (8). The eluate containing the DPN<sup>+</sup> was lyophilized, the residue was dissolved in water, and an aliquot containing 23.0 μmoles of DPN<sup>+</sup> was hydrolyzed by the addition of DPNase as previously described (1). When the hydrolysis was complete, 100 mg of nicotinamide were added as a diluent and the nicotinamide was separated by benzene extraction and analyzed for D (1).

*Experiments 2 and 3*—Though the quantities of reagents used differed somewhat the procedure used was the same as that of Experiment 1, except that 2.5 ml of the solution of glyceraldehyde-3-phosphate in D<sub>2</sub>O (see under "Materials") were used instead of FDP, aldolase was omitted, and in Experiment 3 muscle dehydrogenase was used instead of yeast enzyme. The dilutions, which were different in the various experiments, are indicated.

\* Analyzed as phenacyl lactate

aldolase, and in Experiment 2 DL-glyceraldehyde-3-phosphate was added directly. Experiment 3 was similar to Experiment 2 except that muscle dehydrogenase was used instead of the yeast enzyme.

The results of Table I show that 80 to 90 per cent of the extra H in the reduced DPN must have been derived from the triose phosphate. The reaction catalyzed by both muscle and yeast triosephosphate dehydrogenase obviously proceeds largely with direct transfer of hydrogen. However, the amount of D which was derived from the medium was larger in these experiments than in the case of any of the previous enzyme reactions.

TABLE II  
*Steric Specificity for DPN*

Experiment No	Dehydrogenase source	Lactate*				Nicotinamide			
		Atom per cent excess D found	Dilution factor	Atom D per molecule	$\frac{D \text{ in lactate} \times 100}{D \text{ in nicotinamide-4-}d\text{-DPN}^+}$	Atom per cent excess D found	Dilution factor	Atom D per molecule	Atom D per molecule (corrected)†
					<i>per cent</i>				
1	Yeast	0.051	20.6	0.126	93	0.0195	12.25	0.014	0.00
2	Muscle	0.099	39.7	0.472	99	0.047	20.6	0.058	0.00
3	"	0.097	40.6	0.473	99	0.034	32.7	0.067	0.016

The procedure used for these experiments was essentially the same as that described for Experiment 1 of Table I, except that the reaction mixture was made up in H<sub>2</sub>O instead of D<sub>2</sub>O and the lyophilization to remove D<sub>2</sub>O was omitted. The yeast triosephosphate dehydrogenase used in Experiment 1 was the same as that employed in the experiments of Table I. A commercial preparation of muscle triosephosphate dehydrogenase was used in Experiments 2 and 3.

\* Analyzed as phenacyl lactate.

† Corrected for amount of DPN<sup>+</sup> found by enzymatic analysis to be left in solution after termination of the reduction of DPN by triosephosphate dehydrogenase.

examined. It is of interest to inquire whether the D enters the DPN directly from the medium, or whether it first becomes incorporated into the triose, to be subsequently transferred to the DPN. To answer this question it is necessary to know the steric specificity of the dehydrogenase-catalyzed hydrogen transfer to DPN. This was determined in a second set of experiments, in which DPN<sup>+</sup> containing D in the 4 position of the nicotinamide ring (nicotinamide-4-*d*-DPN<sup>+</sup>) was reduced by triose phosphate in a medium of H<sub>2</sub>O. The remainder of the reaction sequence was identical with that of the first set of experiments, lactate and nicotinamide were again isolated and analyzed for D.

The results of the second set of experiments are given in Table II. In all of these experiments, triose phosphate was generated from FDP by the action of aldolase. Experiment 1 was carried out with yeast dehydro-



genase Experiments 2 and 3 were carried out with the enzyme prepared from muscle The nicotinamide-4-*d*-DPN<sup>+</sup> used in Experiment 1 contained 0.136 atom of D per molecule, the nicotinamide-4-*d*-DPN<sup>+</sup> used in Experiments 2 and 3 contained 0.477 atom of D per molecule

The results given in Table II show that in each case the enzymatic reduction of nicotinamide-4-*d*-DPN<sup>+</sup> by triose phosphate resulted in the formation of a DPND which transferred all of its D to lactate on enzymatic reoxidation by pyruvate The reduced DPN must therefore have been  $\alpha$ -DPND, and this could only have been formed by the transfer of H from triose to the  $\beta$  side of the nicotinamide ring of DPN

The significance of the results given in Table I may now be reassessed The results of the second set of experiments indicate that any D transferred to DPN from triose must be left in the nicotinamide The amount of D found in the nicotinamide was 0.06 atom per molecule in each experiment, whether glyceraldehyde-3-phosphate was added as such or generated from FDP

Clearly, the aldolase reaction does not lead to incorporation in the triose of any D which may be transferred to DPN The most likely source of entry of D in the nicotinamide is probably not the triose, but the exchange which occurs when DPN<sup>+</sup> is heated in D<sub>2</sub>O There is always a little DPN<sup>+</sup> left at the end of the reduction (Equation 1), and this DPN<sup>+</sup> may acquire D from the medium when the enzyme is heat-inactivated Such D would probably be in the 2 position of the nicotinamide This inference is based on the demonstration by San Pietro (11) that D is incorporated into position 2 of the nicotinamide ring of DPN<sup>+</sup> on exposure to alkaline pH in D<sub>2</sub>O Dr Sidney P. Colowick has also informed us that a similar incorporation of D occurs when DPN<sup>+</sup> is heated in D<sub>2</sub>O at neutral pH Finally the amount of DPN<sup>+</sup> in solution at the time of heat inactivation is sufficient to explain the entrance of D into nicotinamide by this route

The presence of D in the lactate in the first set of experiments cannot be so readily explained The fact that this amount varied from 0.04 to 0.13 atom of D per molecule may be taken as an indication that the incorporation of D into the  $\alpha$  position of DPNH is not a necessary part of the oxidation-reduction reaction The two dehydrogenases appear to differ in the amount of D introduced, the yeast enzyme giving an average exchange of 10 per cent in the first set of experiments, which agrees very well with the amount of D lost in the yeast enzyme experiment of Table II The exchange with the muscle enzyme is only about 4 per cent in the experiment in D<sub>2</sub>O, and the data of Table II are not sufficiently accurate to exclude such a possible loss in the experiments with nicotinamide-4-*d*-DPN<sup>+</sup>

An experiment was performed to see whether the yeast enzyme could cause an exchange of D into DPN<sup>+</sup> in the absence of substrate The con

ditions were the same as those for the other experiments except that no tiiose or FDP was added but 24 mg of dehydrogenase were used and the incubation time was 2 hours, *i e* six times longer than the average incubation time of the experiments of Tables I and II. The enzyme was inactivated by the adjustment of the pH to 2 and the DPN<sup>+</sup> was isolated by acetone precipitation and analyzed for non-exchangeable D. The 0.03 atom per molecule found is quite insufficient (in view of the large amount of enzyme and the long incubation time) to account for the D introduced into lactate in the experiments of Table I. The final alternative is that the dehydrogenase causes an exchange of D into the  $\alpha$ -para position of DPNH. It is felt that the data suggest this possibility, though they do not prove it. Further experimental investigation of this possibility is complicated by the fact that tiiosephosphate dehydrogenase causes the conversion of DPNH to DPNH-X (12, 13). Though the rate of DPNH-X formation is much slower than the oxidation-reduction reaction, the quantities of enzyme used in the present experiments were large enough to cause a measurable conversion of this sort.

In conclusion, it is worth noting that both of the DPN-linked aldehyde dehydrogenases tested to date have shown  $\beta$  specificity for DPN, whereas four out of five alcohol dehydrogenases have shown  $\alpha$  specificity. The alcohol dehydrogenases tested include yeast alcohol dehydrogenase (14), lactic dehydrogenase (15), malic dehydrogenase (10), and D-glyceric acid dehydrogenase,<sup>2</sup> all of which show  $\alpha$  specificity, and  $\beta$ -hydroxysteroid dehydrogenase which shows  $\beta$  specificity (16). Were it not for the results with the  $\beta$ -hydroxysteroid dehydrogenase, one might be tempted to generalize that alcohol dehydrogenases show  $\alpha$  specificity, whereas aldehyde dehydrogenases show  $\beta$  specificity.

A possible significance of the different steric specificities observed may lie in the fact that a coupled reaction between two DPN-linked dehydrogenases might be favored if the two enzymes have opposite steric specificities for DPN. It is of interest in this connection that Cori *et al* (17) have found that DPN bound to tiiosephosphate dehydrogenase can be reduced or oxidized by the lactic dehydrogenase reaction under circumstances such that the reaction had to be attributed to collisions between protein molecules.

The geometry of the reduced nicotinamide ring poses a number of interesting questions. The construction of Stuart and Briegleb models shows that the 2 hydrogen atoms in the 4 position are not oriented in the same way with relation to the plane of the ring. 1 of the hydrogen atoms lies in the same plane as the hydrogen atoms which are attached to the other carbon

<sup>2</sup> Unpublished experiments

atoms in the ring This may be called the equatorial position The other hydrogen atom protrudes almost at right angles from the ring in what may be called an axial position However, both the  $\alpha$ - and the  $\beta$ -hydrogen atoms may each occupy either an axial or an equatorial position, depending upon the direction in which the ring is "folded" It seems reasonable to suppose that the enzyme places strict requirements on the conformation of the ring during the enzyme reaction, though it is not apparent which conformation obtains in the case of any single enzyme

#### SUMMARY

Deuterium has been used as a tracer to show that the reaction catalyzed by D-glyceraldehyde-3-phosphate dehydrogenase from both muscle and yeast proceeds largely by way of direct transfer of hydrogen between substrate and the  $\beta$ -4 position of the nicotinamide ring of DPN

*Addendum*—After this paper was submitted for publication, a paper of Nygaard and Rutter (18) appeared, with a confirmation and extension of the findings of Corn et al (17) Nygaard and Rutter studied the reactions of several dehydrogenases with DPN bound to triosephosphate dehydrogenase They concluded that their result virtually eliminate the obligatory intermediation of free DPN in such reactions Of the four dehydrogenases studied by Nygaard and Rutter, two (*i e* lactic dehydrogenase and yeast alcohol dehydrogenase) have also been examined with the aid of deuterium and have been shown to have  $\alpha$  specificity for DPN (14, 15) This provides additional evidence for the working hypothesis that coupling is facilitated between DPN-linked dehydrogenases of opposite steric specificities If one assume that hydrogen is always transferred to and from an axial position on the pyridine ring, then the  $\beta$ -hydrogen should be axial when DPN is reduced by a dehydrogenase reaction of  $\beta$  specificity (*e g* triosephosphate dehydrogenase), whereas the  $\alpha$  hydrogen should be axial when the DPNH is oxidized by a dehydrogenase reaction of  $\alpha$  specificity A shift in conformation of the pyridine ring, not necessarily accompanied by complete dissociation of the molecule, may enable DPNH bound by triosephosphate dehydrogenase to be oxidized by pyruvate in the presence of lactic dehydrogenase (or by acetaldehyde in the presence of alcohol dehydrogenase)

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# STUDIES ON SUCCINIC DEHYDROGENASE

## II ISOLATION AND PROPERTIES OF THE DEHYDROGENASE FROM BEEF HEART\*

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During the past 2 years succinic dehydrogenase has been isolated from animal tissues as a soluble, essentially homogeneous protein. It has been shown to be a ferroflavoprotein with an unusually tightly bound flavin component, and evidence has been presented for the identity of the enzyme with "fumaric hydrogenase" (1-5). The first paper in this series (6) reported methods for the assay of the primary dehydrogenase and for its extraction in soluble form from a variety of animal tissues and from microorganisms. The present paper deals with the purification of the dehydrogenase from beef heart mitochondria and surveys some of its salient catalytic properties and protein characteristics.

### *Materials and Methods*

Phenazine methosulfate was synthesized by a modification<sup>1</sup> of the method of Dickens and McIlwain (7). Antimycin A and BAL<sup>2</sup> were the kind gifts of Dr. Frank M. Strong and Dr. Henry A. Lardy, respectively. The calcium phosphate gel was an aged preparation (1 to 3 months old) (8), and all the other reagents were commercial preparations of high purity. Glass-distilled water was used throughout this work. Double distilled water from commercial block tin-lined stills or reservoirs inactivated the enzyme rapidly and irreversibly, although it was found suitable for mitochondrial preparations after passage through Dowex 50 resin, H<sup>+</sup> cycle.

Total and inorganic iron were estimated by an unpublished modification of the *o*-phenanthroline method (9), elaborated by Dr. H. Beinert of the

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<sup>1</sup> Mimeographed copies of the procedure are available upon request.

<sup>2</sup> The following abbreviations are used: BAL, 1,2-dithiopropanol, FMN, flavin mononucleotide, FAD, flavin adenine dinucleotide, Tris, tris(hydroxymethyl)amino-methane.

Institute for Enzyme Research<sup>1</sup> Spectrographic analyses were performed by the American Spectrographic Laboratories, San Francisco, and lipase acid was kindly determined by Dr W Razzell and Dr I C Gunsalus For electrophoretic analyses and diffusion constants, the Perkin-Elmer model No 38-A apparatus was employed Sedimentation velocity was measured in a Specialized Instruments Corporation analytical ultracentrifuge The dehydrogenase was assayed as previously described (6)

1 unit of succinic dehydrogenase activity is defined as 1 c mm of O<sub>2</sub> uptake per minute under standard assay conditions, and specific activity is defined as units per mg of protein Protein was determined by the biuret method (10) with the following coefficients (optical density at 540 mμ, 1 cm light path, given by a solution of 1 mg of protein per 3 ml of reaction mixture in the presence of 1.5 ml of biuret reagent) first (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitate, 0.095, gel eluate and all stages thereafter, 0.110 These factors were determined on thoroughly dialyzed preparations of known dry weight In particulate preparations and samples containing Tris buffer, protein was measured by dry weight

## Results

### *Isolation of Dehydrogenase*

The use of mitochondria instead of whole tissue for the isolation of the dehydrogenase offers the advantage of a highly concentrated source material, free from many interfering substances which would be difficult to remove from soluble enzyme preparations Their use, however, also entails at least two disadvantages First, the isolation of mitochondria in large quantity and of constant composition is not a simple problem Second the success of the fractionation of the soluble enzyme, after extraction from mitochondrial material, depends primarily on the quality of the mitochondria Since the dehydrogenase occurs in a considerably higher stage of purity in mitochondrial extracts than in those of whole tissues, minor variations in the isolation of the particles may result in contamination with particles whose proteins later interfere with the fractionation of the dehydrogenase in soluble extracts The procedure for the isolation of mitochondria has been modified from a large scale adaptation (11) of the procedure of Schneider (12)

*Preparation of Beef Heart Mitochondria*—Fresh beef hearts from young prime grade cattle, quartered and chilled in ice at the slaughterhouse, were thoroughly freed from fat and connective tissue and passed through a meat grinder (all operations at 3–5°) Lots of 400 gm were immediately blended with 1200 ml of sucrose-phosphate (85 gm of sucrose and 1.85 gm of

$K_2HPO_4$  per liter) in a special, high capacity blender for 45 seconds. The blender<sup>3</sup> was of the overhead type, designed to fit a 7800 ml stainless steel beaker, equipped with a 0.2 horsepower, 18,000 r p m motor, a shaft 25 cm long, and a three-pronged blade which was kept extremely sharp. The pH was adjusted to 8.6 to 8.8 by the addition of 5 to 5.3 ml of 6 N KOH. The homogenate was immediately centrifuged for 10 minutes at 1800 r p m in the International Equipment Company refrigerated centrifuge No. SR-3 by using the No. 632 cups ( $1000 \times g$  at the bottom of the tube, two and one-half blendings per centrifugation). The supernatant fluid was decanted through a double layer of cheese-cloth, care being taken not to disturb the sedimented nuclei, and, after dilution with 7 liters of 0.9 per cent KCl, the mitochondria were collected in the Sharples centrifuge (50,000 r p m, flow rate of 400 to 450 ml per minute). The Sharples bowl was changed after the collection of mitochondria from 2000 gm of minced heart. The sedimented layer, after resuspension by homogenization in a Waring blender in twice its volume of a solution containing 48.1 gm of sucrose, 6.6 gm of KCl, and 0.45 gm of anhydrous disodium succinate per liter, was frozen overnight.

*Preparation of Acetone Powder*—Treatment of the mitochondrial suspension with *tert*-amyl alcohol in the cold extracts a certain amount of protein material without bringing succinic dehydrogenase into solution and thereby simplifies the subsequent purification of the dehydrogenase.<sup>4</sup>

The thawed mitochondrial suspension was briefly blended at low speeds to assure even resuspension, and 0.111 times its volume of *tert*-amyl alcohol was added. After standing 1 hour at 0° with occasional stirring, for each liter of the mitochondrial suspension 10 ml of 0.5 M  $K_2HPO_4$  were added, and the suspension was centrifuged for 15 minutes at 21,000 r p m ( $59,000 \times g$  at the bottom of the tube) in the No. 21 rotor of the Spinco model L ultracentrifuge. The clear yellow supernatant solution was carefully decanted and the residue from each five Spinco tubes was homogenized in the Waring blender with 800 ml of acetone at -10°. The contents of two Waring bowls were stirred with an additional 4 liters of acetone for 5 minutes and then rapidly filtered by suction through a 31 cm Buchner funnel. The moist filter cake was washed with 500 ml of acetone, resuspended in 500 ml of acetone, blended, and filtered again, and the filter cake copiously washed with ether. Residual solvent was removed by spreading the filter cake on heavy paper in the cold room before a fan, and was then dried in a high vacuum at room temperature for 30 minutes. The

<sup>3</sup> The blender was designed by Mr. W. Handrow of the Institute for Enzyme Research. Blueprints are available from the authors upon request.

<sup>4</sup> Treatment with tertiary amyl alcohol is an adaptation of a step in the procedure of Green *et al.* (13) but serves a different purpose here.



resulting light tan powder weighed 40 to 60 gm per 12 kilos of heart mince

*Extraction of Dehydrogenase*—The yield of enzyme on extraction is a function of the efficiency of the blending and stirring and varies from 80 to 100 per cent

A 2 per cent suspension of the acetone powder in 0.06 M Tris buffer, pH 8.9 (pH at room temperature), was first blended at 0° for 45 seconds and then vigorously stirred for 30 minutes. The clear or slightly opalescent yellowish solution obtained on centrifugation at or above  $4000 \times g$  for 25 minutes contained the dehydrogenase in soluble form

*First Protamine and  $(\text{NH}_4)_2\text{SO}_4$  Precipitations*—Treatment of the extract with protamine results in considerable purification and in removal of impurities which appear to combine with the enzyme and to render it highly labile. When used in excess, protamine precipitates the dehydrogenase. For best yield and purity, it is advisable to determine for a given acetone powder the amount of protamine which gives 60 to 70 per cent precipitation of protein, measured by light absorption at  $280 \mu$ , and not more than 20 to 35 per cent precipitation of the enzyme, the usual range being 4.5 to 5 ml of 0.3 per cent protamine sulfate per 100 ml of extract

The extract obtained above was treated with 4.5 ml of 0.3 per cent protamine sulfate in 0.03 M phosphate, pH 7.6, and after being stirred for 10 minutes, it was centrifuged at moderate speed to give a clear yellowish supernatant fluid, which contains most of the enzyme, and a heavy brown pellet. The solution was brought to 0.50 saturation with solid  $(\text{NH}_4)_2\text{SO}_4$ , stirred for 30 minutes, and centrifuged for 25 minutes at  $4000 \times g$ . The precipitate containing 40 to 50 per cent of the activity of the first extract was resuspended in a minimal volume of 0.005 M phosphate, pH 7.6, and dialyzed for 1 hour against a large volume of the same buffer and then for 1 hour against 0.002 M phosphate, pH 7.6, in casings of  $\frac{1}{4}$  inch diameter. The precipitated protein was removed by brief centrifugation at  $18,000 \times g$ , yielding a deep brown solution of the enzyme

*Second Protamine and  $(\text{NH}_4)_2\text{SO}_4$  Precipitations*—After determination of the protein content of the dialyzed enzyme by the biuret method, the solution was diluted to a concentration of 5 mg per ml with 0.06 M Tris buffer, pH 8.9, and stirred with 5.5 ml of 0.3 per cent protamine sulfate per 100 ml of enzyme for 10 minutes. Any precipitate formed was removed by brief centrifugation at  $10,000 \times g$  and the supernatant solution was brought to 0.5 saturation with respect to  $(\text{NH}_4)_2\text{SO}_4$ . After 20 minutes stirring and 25 minutes centrifugation at  $12,000 \times g$ , the almost colorless supernatant solution was discarded and the precipitate, which redissolved readily in a small amount of 0.005 M phosphate buffer, pH 7.6, was dialyzed in  $\frac{1}{4}$  inch casings against a large volume of the same buffer for 2 hours and then for

an additional 2 hours against 0.002 M phosphate, pH 7.6. The clear amber solution was kept frozen overnight. Continuation of the dialysis overnight resulted in slight loss of activity. The yield of enzyme in this step was 70 to 80 per cent.

*Treatment with Calcium Phosphate Gel*—After dilution of the solution to a protein concentration of 10 mg per ml (biuret method), it was stirred with 0.3 mg of calcium phosphate gel per mg of protein for 15 minutes. The gel was centrifuged and discarded and the supernatant solution, after redetermination of its protein content with use of a biuret coefficient of 0.095, was cautiously adjusted to pH 5.2 with 0.5 M acetic acid and stirred with 1.1 mg of calcium phosphate gel per mg of remaining protein. The gel, containing over 90 per cent of the remaining enzyme, was centrifuged and then eluted twice by homogenization and 15 minutes stirring with 0.3 M phosphate, pH 7.6. The volume of eluent, per gm of protein at the beginning of this step, was 50 ml in the first and 33.3 ml in the second elution. After brief centrifugation, the nearly colorless gel was discarded. Yield of enzyme, about 60 to 70 per cent.

*Third  $(\text{NH}_4)_2\text{SO}_4$  Precipitation*—The eluate was treated dropwise with saturated, neutral  $(\text{NH}_4)_2\text{SO}_4$  solution to give 0.30 saturation. After 20 minutes stirring and centrifugation, the precipitate was discarded and the supernatant layer was brought to 0.46 saturation by further addition of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution. After 25 minutes stirring, the precipitated enzyme was centrifuged at or above  $12,000 \times g$ , redissolved in a minimal volume of 0.005 M phosphate-0.1 M NaCl buffer, pH 7.6, dialyzed at least 2 hours against the same buffer, and then frozen. Yield of enzyme, 50 to 55 per cent.

*Ultracentrifugal Separation*—In good preparations the enzyme was about 70 per cent pure at this stage and was devoid of colored impurities. The remaining impurity, which was almost entirely a light weight protein component, could be removed by fractional ultracentrifugation at the expense of some loss of enzyme. The solution from the last step was adjusted to a protein concentration of 10 to 15 mg per ml (biuret factor = 0.110) and centrifuged in 3 ml tubes, fitted by means of special thin bottom Teflon microadapters into the No. 40 rotor of the Spinco model L ultracentrifuge, for 20 minutes at  $144,000 \times g$  (at the bottom of the tube). The clear solution was separated from the slight film of denatured protein and the enzyme was recentrifuged in the same equipment for 4.5 hours. The colorless upper layer and the thin layer immediately over the pellet were removed and united, and the pellet was redissolved in any convenient buffer. In general, the ratio of enzyme in pellet and supernatant fluid was about 4:1.

Table I summarizes the purification procedure, based on 60 gm of acetone powder. This amount of starting material contains 250,000 to 300,000

units of enzyme, but a deviation of 10 to 15 per cent in yield and specific activity from the values given is not unusual. Some inactivation frequently occurred during ultracentrifugation since, although the total protein was satisfactorily recovered in the pellet and the supernatant fluid, some 20 to 30 per cent of the activity could not be accounted for. In such instances the activity observed in the final product was corrected for inactivation by the factor (total protein recovered)/(total activity recovered in ultracentrifugation).

TABLE I  
*Purification of Succinic Dehydrogenase*

Step	Total units	Specific activity*
Acetone powder suspension	280,000	3
Tris extract	256,000	10
After 1st (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> step	134,000	147
“ 2nd “ “	92,600	174
Gel eluate	64,800	200
After 3rd (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> step	35,100	230
“ differential ultracentrifugation	27,200	302†

\* Based on dry weight in the first two stages and on biuret coefficient of 0.110 thereafter

† Corrected for inactivation during ultracentrifugation

### *Electrophoresis, Sedimentation, and Molecular Weight*

In the pH range of 7.0 to 7.9 in phosphate and Tris buffers of 10 mM strength = 0.1, the enzyme migrated as a single boundary in the Tiselius apparatus (5). In preparations with a specific activity of 300, the total impurity detected was of the order of 4 to 7 per cent. At 0° in 0.1 M NaCl 0.005 M phosphate, pH 7.1, the observed mobility was  $2.8 \times 10^{-5}$  cm<sup>2</sup> volt<sup>-1</sup> sec<sup>-1</sup>.

Examination<sup>6</sup> of several preparations in the analytical ultracentrifuge at protein concentrations from 1 to 1.5 per cent revealed the presence of a single sedimenting boundary (Fig. 1) with an  $s_{20}$  value of 6.5 S. From the latter figure and from a preliminary measurement of the diffusion constant ( $D_{20} = 4.2 \times 10^{-7}$  cm<sup>2</sup> sec<sup>-1</sup>), a molecular weight of 150,000 has been calculated. This value is probably too low since the sedimentation velocity is not corrected to zero protein concentration and an estimated 8 per cent of impurity of low molecular weight was known to be present in the

<sup>6</sup> We are grateful to Dr. D. Basinsky of the Department of Laboratories, Henry Ford Hospital, for permission to use the Spinco analytical ultracentrifuge.

sample used in the diffusion measurement, both of which factors would tend to lower the calculated molecular weight

The molecular weight from light scattering data is about 220,000, as kindly determined by Dr Robert Steiner. The most accurate estimate of the molecular weight came from analyses of the iron content of the dehydrogenase. Preparations isolated from *fresh* mitochondrial acetone powders were found to contain 1 gm atom of Fe per 49,000 gm of protein, whereas the dehydrogenase isolated from aged acetone powders (*cf* below) contained 1 gm atom of Fe per 100,000 gm of protein (Table II). The

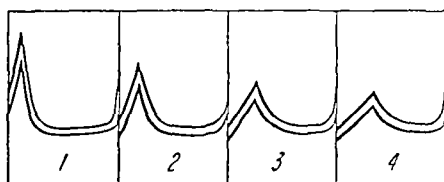


FIG 1 Sedimentation pattern of succinic dehydrogenase in the ultracentrifuge. Protein concentration, 9.1 mg per ml, buffer, 0.1 M NaCl-0.005 M phosphate, pH 7.6, temperature, 4.65°, speed, 59,771 r.p.m., bar angle, 45°. Centrifugation was continued until the sedimenting peak reached the bottom of the cell. Each of the four exposures was spaced 16 minutes apart.

TABLE II  
*Iron Content of Succinic Dehydrogenase*

	Iron content*	Specific activity
Fresh enzyme	1 mole per 49,000 gm	320-330
Enzyme from aged acetone powder	1 " " 105,000 gm	110-115

\* Total iron or iron liberated by cold 5 per cent trichloroacetic acid

minimal molecular weight is, then, 100,000, and the probable provisional value of the molecular weight is 200,000. As will be reported in a later publication<sup>6</sup>, this figure is also in fair agreement with the flavin content of the enzyme (1 mole per 200,000 gm of protein). Thus, the dehydrogenase isolated from fresh starting material contains 4 atoms of Fe per mole of flavin, whereas from aged material an enzyme containing 2 atoms of Fe per mole of flavin may be isolated.

### *Stability*

In the form of the mitochondrial acetone powder, the enzyme has been preserved for periods of 3 to 8 months at -20°. In the purified form the

<sup>6</sup> V Massey, T P Singer, and E B Kearney, to be published

enzyme is decidedly unstable. The inactivation encountered on storage of highly purified preparations at  $-20^{\circ}$  (10 to 20 per cent in 24 hours, 20 to 30 per cent in 5 days) may be due, at least in part, to a gradual loss of iron. With reagents prepared in glass-distilled water, reversible  $-SH$  oxidation appears to play no significant part in the inactivation encountered during purification or on storage, since Versene and glutathione failed to protect the enzyme.

### *Turnover Number*

The specific activity of the best preparations (310 at pH 7.6,  $38^{\circ}$ ,  $0.07M$  succinate) corresponds to a  $Q_{O_2}$  of 18,000. When corrected to maximal substrate concentration ( $V_{max}$ ), the  $Q_{O_2}$  is 20,000. With a molecular weight of 200,000, this is equivalent to 3000 moles of succinate oxidized per mole of enzyme per minute, a value comparable to the turnover number of other flavoproteins. The concentration of phenazine methosulfate used here (2 to 3 mg per 3 ml of reaction mixture) gives apparent saturation with respect to the dye, since higher concentrations are somewhat inhibitory. The true  $V_{max}$ , calculated by the double reciprocal method, is some 12 to 15 per cent higher in *fresh* preparations of the enzyme. Consideration of this additional correction gives a  $Q_{O_2}$  of 23,500 and a turnover number of 3530. It may be further calculated that the acetone powder used as starting material for the isolation of the soluble enzyme contains about 1 gm of succinic dehydrogenase per 60 gm.

### *Iron Content*

The total iron content of the dehydrogenase (Table II) is liberated as inorganic iron by boiling or by denaturation of the enzyme with trichloroacetic acid. No hemin iron was detected in the preparation at any stage. Comparison of the color obtained in the *o*-phenanthroline reaction (9) in the presence and absence of reducing agents showed that the iron is present in the enzyme entirely in the ferrous form.

Since the acid-labile iron follows the activity closely through most of the purification and since the ratio of specific activity to iron is constant in the two types of preparation (4-Fe and 2-Fe type, Table II) with either phenazine methosulfate or ferricyanide as terminal electron acceptors, it appears that both of these oxidants accept electrons at the level of the iron and not at the level of the flavin. The 4-Fe and 2-Fe enzymes behaved identically in most cases, *i.e.* behavior in the fractionation procedure, molecular weight, electrophoretic mobility, pH optimum, and phosphate requirement, etc., but differed from each other in the following respects. The 4-Fe enzyme is more sensitive to inhibition by substances which chelate with iron than its 2-Fe counterpart, and part of its iron content appears to be

more or less readily lost, these observations will be reported in Paper III of this series. The two types of preparations also show different absorption spectra (Fig 2). It is noteworthy that at wave lengths above 500  $m\mu$ ,

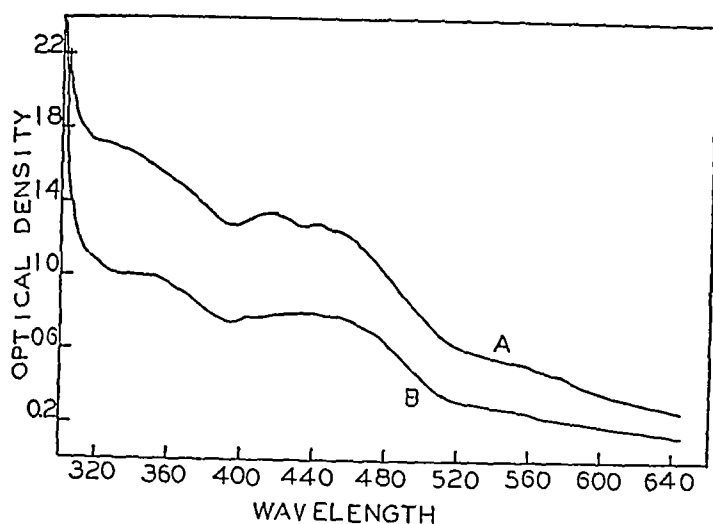


Fig 2 Comparison of the spectra of the 4-Fe (A) and 2-Fe (B) enzymes at a protein concentration of 10.9  $\gamma$  per ml and pH 7.6

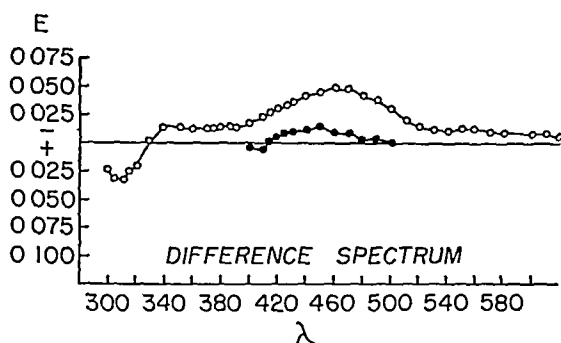


Fig 3 Difference spectrum of the enzyme after reduction by succinate and the inhibition of the process by malonate. 2-Fe enzyme, 4 mg per ml, at pH 7.6. Succinate and malonate concentrations,  $1.25 \times 10^{-2}$  M. Succinate alone bleached 18 per cent of the color at 460  $m\mu$ . O, after succinate, ●, after succinate plus malonate

where the contribution of flavin to the color is negligible and where the non components of other ferroflavoproteins absorb light (14), the ratio of the colors of the two preparations agrees with the ratio of their iron content. Further, at 450  $m\mu$ , at the flavin maximum, the greater part of the color is still due to iron and not to flavin. As shown in Fig 3, succinate causes a partial bleaching of the color, with a maximum centering around 460  $m\mu$ , at this wave length, in the case of the 2-Fe enzyme, about 18 per cent of the

color is bleached by succinate and 63 per cent by hydrosulfite. Malonate competitively inhibits the bleaching by succinate.

### *Effect of pH*

The pH-activity relations of the enzyme are shown in Fig. 4. As previously noted (15), the activity in phosphate buffers is considerably higher than in Tris, imidazole, histidine, or glycylglycine buffers, arsenate does not replace phosphate but inhibits the stimulatory effect of phosphate. The enzyme used in the experiments summarized in Fig. 4 contained appreciable phosphate since it had been dialyzed against 0.005 M phosphate and because, under these conditions, the dehydrogenase binds a considerable

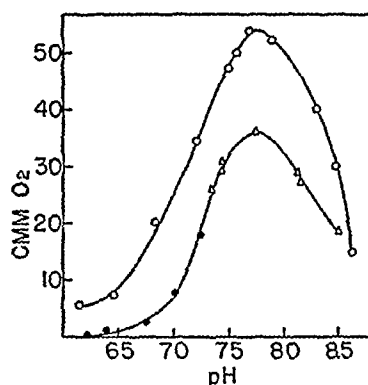


Fig. 4. pH-activity curve of succinic dehydrogenase. Standard assay conditions, except for buffer, as noted. Each vessel contained 0.06 mg. of enzyme (third  $(\text{NH}_4)_2\text{SO}_4$  precipitate) and 150  $\mu$ moles of buffer as follows: O, phosphate; ●, imidazole; Δ, Tris. The pH values marked on the abscissa were measured at 38° in the complete reaction mixture.

amount of inorganic phosphate. The stimulatory effect of added phosphate is nevertheless obvious, for concentrations of added phosphate as high as 0.05 M are required for maximal stimulation. In preparations isolated in the absence of added phosphate, the stimulation is about 3 fold (15). It is seen in Fig. 4 that this effect cannot be explained as a shift of the pH optimum. No requirement for added cations has been observed.

### *Effect of Substrate Concentration*

At pH 7.6, 38°, with phenazine methosulfate as acceptor, the  $K_m$  of succinic dehydrogenase of beef heart for succinate is  $1.3 \times 10^{-3}$  M, in agreement with data in the literature (16) for rat liver succinic oxidase at this pH and temperature, and with values obtained by the authors for Keilin-Hartree preparations of succinic oxidase from heart (38°, pH 7.6, O<sub>2</sub> as the terminal acceptor). At 21°, the  $K_m$  of the purified soluble dehydrogenase

in the presence of phenazine methosulfate is  $5.2 \times 10^{-4}$  M, in agreement with the data of Slater and Bonner ( $4.8 \times 10^{-4}$  M (17)) and Thorn ( $5.1 \times 10^{-4}$  M (18)) obtained at 20–25° with ferricyanide as acceptor. Lower values in the literature (*e.g.*  $1.8 \times 10^{-4}$  M (13), temperature and electron acceptor not stated) may be due to the use of electron acceptors which do not permit the functioning of the dehydrogenase at full capacity. The  $K_m$  for succinate is remarkably constant in succinic dehydrogenase from various sources in the presence of acceptors which react with the primary dehydrogenase. Thus, the  $K_m$  values of the soluble dehydrogenase from *Proteus vulgaris* (6) and from bakers' yeast<sup>7</sup> are  $1.3 \times 10^{-3}$  M and  $1.05 \times 10^{-3}$  M at pH 7.6, 38°, with phenazine methosulfate as acceptor.

At pH 7.6 and 38°, the competitive inhibition of the beef heart enzyme by malonate, expressed as the ratio  $K_m/K_i$ , is about 30, at 21° the ratio is about 21. The values of this ratio in the literature range from 3 to about 60 (see Thorn (18)), depending upon the temperature, type of preparation, and electron acceptor used, as fully discussed elsewhere (18). The lowest ratios reported by Thorn are extrapolated values, based on the use of methylene blue as an acceptor, which introduces a factor of uncertainty in the calculations, since methylene blue cannot react directly with the dehydrogenase (6, 17). Unlike the  $K_m$ ,  $K_m/K_i$  for malonate apparently varies with the source of dehydrogenase, since in this laboratory a value of 93 was observed<sup>7</sup> for the yeast enzyme at 38°, in satisfactory agreement with the preliminary data of Krebs *et al.* (19). The  $K_m/K_i$  for fumarate at 38° is 0.68 in the case of the beef heart enzyme. Oxalacetate is a potent competitive inhibitor, as in mitochondrial preparations, but the inhibition has not yet been quantitatively characterized.

### *Effect of Inhibitors*

In agreement with observations on the particulate enzyme (20, 21), the dehydrogenase is unaffected by treatment with cyanide ( $5 \times 10^{-3}$  M), antimycin A (5  $\gamma$  per ml), or BAL ( $10^{-3}$  M, followed by dialysis to remove excess thiol), all of which substances are thought to inactivate the succinoxidase system above the level of the dehydrogenase. At the concentration used in the standard assay system ( $2.4 \times 10^{-3}$  M), phenazine methosulfate inactivates the enzyme 90 per cent or more if added to the enzyme prior to the substrate, probably by oxidation of the —SH groups, previous addition of succinate prevents this effect (6). In further agreement with the behavior of particulate preparations, the enzyme is exceedingly sensitive to sulfhydryl reagents, particularly of the mercaptide-forming group (Table III). Even in a relatively crude preparation such as represented

<sup>7</sup> T P Singer, N Zastrow, V Massey, and E B Kearney, to be published



in Table III,  $1 \times 10^{-6}$  M *p*-chloromercuribenzoate gave 90 per cent inhibition in the presence of 150  $\gamma$  of enzyme, in more purified preparations the sensitivity to this reagent increased further. As in particulate preparations (22), previous addition of succinate protected the dehydrogenase considerably from the effect of —SH inhibitors (Table III). Cyanide, at a concentration of  $1 \times 10^{-3}$  M, reversed the inhibition by *p*-chloromercuribenzoate completely, as did treatment with  $10^{-3}$  M glutathione, followed by brief dialysis to remove the unchanged thiol. The ready reactivation of the enzymatically inactive *p*-chloromercuribenzoate compound of the de-

TABLE III  
*Effect of —SH Inhibitors on Succinic Dehydrogenase*

Experiment No	Treatment	Inhibition per cent
1	<i>p</i> -Chloromercuribenzoate, $1 \times 10^{-6}$ M	91
2	Same as Experiment 1, but succinate added before inhibitor	33
3	<i>p</i> -Chloromercuribenzoate, $1 \times 10^{-6}$ M, followed by 3.5 hrs dialysis	90
4	Same as Experiment 3, but $1 \times 10^{-3}$ M HCN in assay	0
5	“ “ “ 3, “ $3 \times 10^{-3}$ “ glutathione added before dialysis	0
6	<i>o</i> -Iodosobenzoate, $2 \times 10^{-4}$ M	100
7	Iodoacetamide, $1 \times 10^{-3}$ M	71

Conditions: Enzyme, 150  $\gamma$  at first  $(\text{NH}_4)_2\text{SO}_4$  stage. Standard assay conditions except that, in Experiments 1 to 3 and 5 to 7,  $1 \times 10^{-3}$  M 8-hydroxyquinoline was used to overcome the effect of  $\text{H}_2\text{O}_2$  produced in the reaction in the place of HCN (6). In Experiment 5, 10 minutes contact was allowed between enzyme and mercurial derivative prior to addition of the thiol.

hydrogenase by the HCN present in the standard assay mixture permitted the fractionation of the enzyme throughout the procedure outlined above and the mercurial derivative. It could be shown by this means that the mercurial derivative and the active enzyme could be purified by the same procedure, with no apparent differences in solubility and stability between the two. The inhibition of the dehydrogenase by iron-chelating substances will be discussed in Paper III.

#### *Reaction with Various Acceptors*

The relative efficiency of various electron acceptors is the same for the 2-Fe and 4-Fe enzymes at the highest stage of purity as in crude, soluble preparations (6). With the turnover of succinate in the presence of excess phenazine methosulfate taken as 100, the rate with ferricyanide is 30 in the presence of serum albumin as a protective agent, 5 to 25 in the absence

of albumin, all other dyes tested and cytochrome *c* fail to act as electron acceptors at a significant rate (A very slow reaction with methylene blue has been observed spectrophotometrically by Di Massey in this Laboratory, which may be a direct reaction with the enzyme, unlike the rapid reaction observed with particulate preparations) Molecular  $O_2$  reacts slowly (1/5000th the rate of phenazine methosulfate), accepting electrons directly from the flavin (5) The high specificity of the dehydrogenase for electron acceptors when acting in the forward direction is all the more curious, since in the reverse reaction the reduced forms of riboflavin, FMN, FAD, and diethylsaffranin all act efficiently as electron donors to fumarate In these instances the reaction with the dyes occurs at the level of the flavin and not of the iron, whereas in the oxidation of succinate both phenazine methosulfate and ferricyanide accept electrons from the iron moiety

#### DISCUSSION

The demonstration that the oxidation of succinate to fumarate is catalyzed by a single discrete protein molecule, whose catalytic action shows all the important features usually associated with succinic dehydrogenase action, permits the identification of this protein as succinic dehydrogenase The differences in the behavior of the highly purified, soluble dehydrogenase and particulate preparations are the relatively high specificity of the former for electron acceptors, which is a consequence of the complete removal of the various electron transport components, and the activation of the soluble enzyme by phosphate, possible reasons for which have been discussed elsewhere (15) It is evident from these considerations and from the data presented in the preceding paper that preparations of the enzyme capable of oxidizing succinate efficiently with methylene blue, brilliant cresyl blue, tetrazolium, or indophenol dyes as acceptors must contain additional factors, possibly members of the original cytochrome chain, which catalyze the reaction between the primary dehydrogenase and these dyes

The classical studies of Keilin and Hartree (23) led to the view that the succinic oxidase system, even in cell-free preparations, exists as an organized respiratory system wherein succinic dehydrogenase and the various members of the cytochrome system are bound in a functional, organized unit This view received further support from the successful isolation of the succinic dehydrogenase-cytochrome system as a particulate entity by Green *et al* (13, 24) Subsequent to the authors' report of the isolation of the soluble dehydrogenase (25), Green and coworkers explored the possibilities of alternative procedures for the isolation of the dehydrogenase These included treatment of their particulate preparations with trypsin in the presence of bile salts and alkaline degradation thereof (24, 26, 27)

The various preparations thus obtained were reported to contain succinic dehydrogenase activity in solution, but analysis revealed significant differences between them and succinic dehydrogenase. Thus, the products of proteolysis contained 1 to 4 moles of hemin per mole of flavin, whereas the alkaline degradation product yielded 1 atom of non-hemin iron (instead of 4)<sup>8</sup> and no hemin per mole of flavin. These preparations further differed among each other and from succinic dehydrogenase in their acceptor specificity. It was concluded apparently that the products mentioned represent different "types" or "species" of succinic dehydrogenase (24, 26, 27). The present authors are not inclined to this view, since neither in the isolation of succinic dehydrogenase from animal tissues nor from bakers' yeast<sup>7</sup> was any evidence encountered for the existence of more than one type of succinic dehydrogenase nor for a change in its acceptor specificity in the course of purification. Alternative explanations have not been precluded. Since none of the preparations of Green *et al* have been isolated in a state approaching homogeneity, the possibility remains that they contain, besides succinic dehydrogenase itself, contaminating hemoproteins. Another possibility, favored by the present authors, is that the degradative procedure used by these workers may be incomplete, breaking the succinoxidase chain at various points above the level of the dehydrogenase, liberating fragments which indeed contain succinic dehydrogenase, but along with other members of the succinoxidase chain, may or may not be structurally attached, possibly by a lipid matrix, to the dehydrogenase itself (28). If this should be true, it would be desirable to avoid confusion by clearly indicating their non-identity with the ferroflavoprotein succinic dehydrogenase.

#### SUMMARY

1 Succinic dehydrogenase has been isolated from beef heart mitochondria as a soluble protein in a state approaching homogeneity by physicochemical criteria. The over-all purification is about 100-fold compared with a mitochondrial acetone powder.

2 The enzyme is a ferroflavoprotein containing 4 atoms of ferrous (non-hemin) iron and a mole of flavin per mole of protein (200,000 gm). The dehydrogenase may be isolated from aged starting material with 2 atoms of iron per mole and half the specific activity.

3 Among the common electron acceptors, only the following function with the dehydrogenase, at the relative rates indicated in parentheses: phenazine methosulfate (100), ferricyanide (39), O<sub>2</sub> (0.02). The first two of these acceptors react via the iron moieties, whereas O<sub>2</sub> seems to react directly with the flavin.

<sup>8</sup> The iron of succinic dehydrogenase is readily labilized by strong alkali.

4 The  $Q_{O_2}$  has been measured as 20,000 and the turnover number as 3000 under the standard assay conditions

5 The properties of the isolated dehydrogenase agree with those previously described for mitochondrial and other particulate preparations of the enzyme, except for properties related to the absence of contaminating hemoproteins. At 38° the pH optimum is 7.7, the  $K_m$  for succinate is  $1.3 \times 10^{-3}$  M at 38° and  $5.2 \times 10^{-4}$  M at 21°. Oxalacetate, malonate, and fumarate are competitive inhibitors. Antimycin A and BAL do not inhibit the dehydrogenase. The dehydrogenase is highly sensitive to sulfhydryl reagents, *p*-chloromercuribenzoate inhibiting it in a reversible manner and the substrate protecting the enzyme from this type of inhibition.

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<sup>8</sup> The iron of succinic dehydrogenase is readily labilized by strong alkali.

# ACTION OF BACTERIOPHAGE ON ISOLATED HOST CELL WALLS\*

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The initial step in the interaction of the tadpole-shaped bacteriophages with their host *Escherichia coli* B is considered to be a reversible adsorption due largely to the formation of electrostatic bonds (1, 2). The bacterial virus is apparently adsorbed tailfirst to the cell (3). Certain chemical groupings on both the bacterial surface and the bacteriophage have been implicated by Tolmach and Puck (4) as participating in this attachment. After the reversible attachment of the virus, an irreversible step occurs, and within a short time the host cell is killed. Subsequently, or perhaps simultaneously, the virus is dissociated into protein and DNA (deoxyribonucleic acid) portions. Most of the viral protein remains as an empty "coat" outside the cell while only the DNA enters the cell (5). This latter process has been termed "injection." The injected DNA apparently initiates the replication of new virus particles.

An early attempt by Cohen (6) to infect intact cells with isolated DNA was unsuccessful. Apparently the protein portion of the virus not only protects the viral DNA and attaches it to the host but also insures its entrance into the host cell. This paper is concerned with quantitative studies of the nature of the invasion process subsequent to attachment of the virus.

In 1951 Weidel (7) devised a method for isolating cell walls from *E. coli* and reported that when these isolated cell walls were incubated with bacteriophage the cell walls were decomposed. This was the first evidence that one of the steps of viral infection involved the alteration of the host cell surface. The use of isotopically labeled cell walls appeared to offer the best means for studying the alteration of the host cell wall. In a preliminary note (8) in 1954, we reported that  $T_2^{+}$  caused the release of  $N^{15}$  from  $N^{15}$ -labeled cell walls. It was also found that, when only a relatively few virus particles (up to fifteen) were adsorbed to a cell wall, the amount of  $N^{15}$  released was proportional to the number adsorbed. Re-

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cently Weidel and Koch (9) have also found that nitrogenous material is released from the cell wall and that the amount depends on the number of virus units adsorbed

It has now been found that viruses other than  $T_2$  will release  $N^{15}$  from cell walls prepared according to Weidel (7) or by the method of Salton and Horne (10). Various other aspects of virus and cell wall interaction such as the effect of temperature, time, and ionic strength have also been investigated. It appears that one of the steps in viral invasion involves the enzyme-like action of a portion of the viral tail on some nitrogenous components in the cell wall.

### *Materials and Analytical Methods*

$T_{21}$ ,  $T_{21}^+$ ,  $T_{41}^+$ ,  $T_5$ ,  $T_{61}^+$ , and  $T_7$  bacteriophage were prepared by infection of *E. coli* B in liquid culture or by confluent lysis on agar plates (11). Nitrogen infectivity measurements were routinely made on all purified preparations (12). Phage assays were carried out by the plaque count technique (13). Cell walls were counted in a Petroff-Hausser counting bacterial chamber with the use of a phase contrast microscope. Sedimentation constants were determined in the Spinco model E ultracentrifuge and electrophoretic mobilities in the Pearson electrophoresis apparatus.

Nitrogen was determined by the micro-Kjeldahl method of Ma and Zuazaga (14).  $N^{15}$  was measured in the mass spectrometer; samples were prepared for analysis by the method of Rittenberg (15).  $P^{32}$  and total phosphorus were determined by methods previously described (16). Total reducing substances were determined (as glucose) by the method of Herbert (17) and hexosamine by a modified Elson and Morgan method (18). F was obtained from the Argonne Cancer Research Hospital as phosphate in weak HCl.  $N^{15}$  (7.7 atom per cent excess) was purchased as  $N^{15}H_4NO$  from the Eastman Kodak Company and distilled into sulfuric acid. Trypsin, lysozyme, and ribonuclease were obtained from Armour and Company and the deoxyribonuclease from the Worthington Biochemical Corporation. This (tris(hydroxymethyl)aminomethane) was obtained from the Sigma Chemical Company.

### *Results*

#### *Preparation and Nature of Bacterial Cell Walls*

*Preparation of  $N^{15}$ -Labeled Cell Walls*—*E. coli* B was grown with aeration at 37° on the following media (gm per liter):  $Na_2HPO_4$ , 10.9;  $KH_2PO_4$ , 10.0;  $MgSO_4$ , 0.2;  $FeSO_4$ ,  $5 \times 10^{-4}$ ;  $CaCl_2$ , 0.01; sodium *L*-malate, 6.6; glucose, 3.35;  $(N^{15}H_4)_2SO_4$ , 1.24, adjusted to pH 7.0. Usually 4 to 5 liter of the above medium were inoculated with a subinoculum of *E. coli* B.

Cells were harvested by centrifugation when the bacterial concentration reached  $1.4 \times 10^9$  cells per ml.

Cell walls were then prepared by using either the procedure of Weidel (7) or that described by Salton and Horne (10). In the Weidel procedure the cells were allowed to autolyze, and were extracted with ethanol and treated with trypsin and lysozyme. The product was then purified by four cycles of differential centrifugation in which material sedimenting within 5 minutes at  $3000 \times g$  in an angle centrifuge was discarded, while the cell walls were sedimented in 30 minutes at  $19,000 \times g$ .

In the Salton-Horne procedure, the harvested cells were suspended in a small volume of 0.9 per cent NaCl and heated at  $75^\circ$  for 50 minutes. The pH was then adjusted to 7.5 with Tris buffer, and 0.4 M  $MgSO_4$  was added so that the  $Mg^{++}$  ion concentration was 0.01 M. The suspension of heated cells was then digested at  $37^\circ$  for 2 hours with a mixture of the following enzymes: deoxyribonuclease (1  $\gamma$  per ml), ribonuclease (1  $\gamma$  per ml), lysozyme (5  $\gamma$  per ml), and trypsin (1 unit per ml). The cell walls were then isolated by differential centrifugation and finally suspended in water.

*Chemical Nature and Physical Properties of Cell Walls*—The Weidel cell walls represent about 10 per cent of the original cell N and the Salton-Horne about 20 per cent of the cell N. Some of the chemical properties of the cell wall preparations are listed in Table I. Comparisons are also shown with values which Weidel (7) and Salton (19) have reported. The data indicate that the preparations contain about 65 to 70 per cent protein. With the assumption that the cell wall protein is of the usual type and has a nitrogen content of 16 per cent, the presence of carbohydrate (9 to 16 per cent) and lipide (10 to 22 per cent) would lower the N content to the observed value of 10 per cent. (The N of the hexosamine is negligible in relation to protein N.) Salton (19) has reported that essentially all the amino acids of the intact host cell are also found in the cell wall. The cell wall carbohydrate is the mucopolysaccharide type found in many bacterial antigens. Hexosamine has been demonstrated in our preparation and in cell walls prepared by Salton. Salton (19) has reported the presence of both glucose and galactose. Recently Weidel and coworkers (20) have also reported the presence of hexosamine and a heptose in the receptor substance for  $T_3$ ,  $T_4$ , and  $T_7$  bacteriophage.

The cell walls contain phosphorus, but it is important to note that there is no RNA (ribonucleic acid) and no DNA in either cell wall preparation. The orcinol test for RNA and the cysteine-sulfuric test for DNA showed that less than 0.1 per cent of either substance could be present. Nucleic acid does not appear to be a constituent of the isolated cell wall.

Although each cell wall preparation studied moved as a single boundary



bacteriophage and the cell walls are almost completely sedimented. The amount of total N and  $N^{15}$  in the final supernatant solution was measured. The amount of  $N^{15}$  liberated by phage-cell interaction was corrected for the amount of  $N^{15}$  found in the control tube containing no virus. This correction was usually of the order of 1 to 2 per cent of the total cell wall  $N^{15}$ .

When  $N^{15}$ -labeled walls prepared according to the Weidel procedure were allowed to interact with six different T phages at the same multiplicity, all except  $T_7$  were well adsorbed (Table II). For this set of experiments the media contained those substances known to provide an efficient en-

TABLE II  
*Interaction of Cell Walls with T Phages\**

Phage	Phage adsorbed	Wall N made non sedimentable	Wall N made non sedimentable per phage
	<i>per cent</i>	<i>per cent</i>	<i>mg</i> $\times 10^{-14}$
$T_{2r}$	99.3	4.4	5.1
$T_{2r}^+$	97.5	3.9	4.5
$T_{4r}^{++}$	99.4	4.3	4.9
$T_6^{\dagger}$	99.9	5.5	6.3
$T_6^+$	97.8	3.2	3.7
$T_7^{\S}$	61.6	0.0	0.0

\* 30 minute incubations at  $37^\circ$  in isotonic-buffered saline, except as noted. Multiplicity = 20. Weidel cell wall Preparation 6.

$\dagger$  0.005 per cent DL-tryptophan added.

$\ddagger$  Incubation in Ringer's solution.

$\S$  Incubation in 0.002 M NaCl.

vironment for infection of intact cells by each bacteriophage.  $T_{2r}$ ,  $T_{2r}^+$ ,  $T_{4r}^{++}$ ,  $T_6$ , and  $T_6^+$  all liberated equivalent amounts of cell wall N.  $T_7$  failed to cause the release of any wall nitrogen although 62 per cent of the added  $T_7$  were inactivated during the incubation. Although  $T_7$  adsorbs well only in media of low ionic strength, it seems possible that a higher salt concentration may be necessary for subsequent reactions during invasion.

An experiment was performed with  $T_{21}^+$  phage which had been osmotically shocked, treated with DNase (deoxyribonuclease), and centrifuged so that a suspension of only the protein coats of the virus was obtained (21). These viral "ghosts" caused the liberation of non-sedimentable N from cell walls. At a calculated multiplicity of twelve "ghosts" per cell wall, each "ghost" caused the liberation of  $5 \times 10^{-14}$  mg of N which is very similar to that liberated by intact  $T_{2r}^+$ . It can be concluded that

al DNA is not necessary for the breakdown of the cell wall, but that al protein alone is responsible

It was also found in these experiments that some viral N was made n-sedimentable upon interaction with the cell wall preparation. Typical values for the amounts of  $T_{21}^+$  N released have been given earlier (8) and ranged from 15 to 38 per cent of the viral N. The identity of the viral

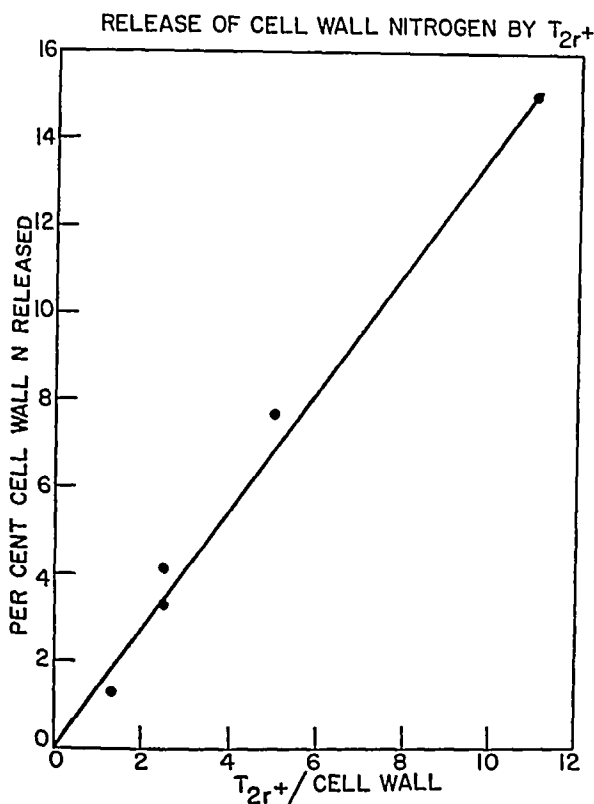


FIG 1 The effect of viral multiplicity on the release of N from cell walls prepared by the Weidel procedure (Preparation 2)

was not closely examined, but on the basis of its phosphorus content and its ultraviolet light adsorption it apparently consists largely of viral A.

*Effect of Viral Multiplicity on Release of Cell Wall N*—In these experiments the results varied with the type of cell wall used and its exact treatment during its preparation. The most consistent results were obtained with Weidel's Preparation 2 (see "Chemical nature and physical properties") when it was used immediately after preparation. In Fig 1 it can be seen that at relatively low multiplicities the amount of cell wall N liberated is proportional to the number of phages adsorbed. The amount of

N released per phage adsorbed was 9 to  $10 \times 10^{-14}$  mg of N or twice that obtained with Weidel's Preparation No 6 (see Table III). Increasing the multiplicity above fifteen phages per cell wall did not increase the amount of N liberated.

Although there was some variation among the preparations in the fraction of N liberated (which depends on the amount of N per cell wall), the maximal amount of cell wall nitrogen liberated even at multiplicities of 150 to 200 was 15 per cent for the Weidel cell walls and 13 per cent for the Salton and Horne cell walls<sup>1</sup>. It appears that at low multiplicities not only

TABLE III  
*Interaction of Bacterial Cell Walls and  $T_2r^+$  Bacteriophage under Various Conditions*

Experiment No	Conditions*	$T_2r^+$ added Cell wall	$T_2r^+$ adsorbed Cell wall	Cell wall N $m_2r^+$ non sedimentable
				per cent
I	T = 4°	280	151	0
	" = 37°	280	245	8.2
II	$\frac{r}{2} = 0.0003$	200	138	0
	$\frac{r}{2} = 0.16$	200	194	4.1
III	0.005 M Versene	40	21	2.2
	No Versene	40	34	2.3

\* Unless otherwise indicated, the reaction mixture had an ionic strength of 0.16 ( $\frac{r}{2} = 0.16$ ) including 0.001 M  $Mg^{++}$  and was incubated at 37° for 30 minutes. In these experiments three different Salton and Horne cell wall preparations were used.

does each phage adsorbed have a limited action on the bacterial surface structure but that only a limited amount of cell wall material can be liberated.

*Effect of Various Conditions on Release of Cell Wall N*—It was found that only 2 to 3 minutes incubation at 37° gave maximal liberation of cell wall N. After the incubation period the tubes were chilled in an ice bath and centrifuged in the cold. With Salton and Horne cell walls (Table

<sup>1</sup> We had previously reported (8) that as much as 74 per cent of the cell wall could be made non-sedimentable by interaction with  $T_2r^+$  at a multiplicity of 187. However, this result was obtained with a cell wall preparation which had been stored at -20° for some months, and the release of similarly large amounts of cell wall N was not observed with unfrozen fresh preparations of either Weidel or Salton and Horne cell walls.

III), both the irreversible adsorption and the liberation of wall N are apparently temperature-dependent. Adsorption of more than half of the added phages was complete at 4° in 20 minutes. However, the interaction at this temperature did not cause liberation of any wall N<sup>15</sup>, while the samples incubated at 37° showed an increase in non-sedimentable wall N.

The ionic requirements for the attachment of T<sub>2</sub>r<sup>+</sup> have been previously reported by Puck and coworkers (1). However, the breakdown of the bacterial surface would not necessarily require the same ionic environment. The results of an experiment to test the ionic requirements of the cell wall breakdown process are shown in Table III.

T<sub>2</sub>r<sup>+</sup> bacteriophages were centrifuged and suspended in distilled water. Salton and Horne cell walls were added to this phage, and the mixture was incubated 30 minutes at 37° in a system to which no electrolyte had been added. Samples were taken for phage and nitrogen analyses. Hypertonic NaCl was added to the remainder of the mixture to bring the ionic strength to 0.16. This was incubated at 37° for a second 30 minutes, when samples for phage and nitrogen analyses were again taken.

The results in Table III show that phage adsorption is decreased in the absence of added ions. Further, there is no release of N from either bacteriophage or cell walls in the absence of added ions, but, when the ionic strength is increased, cell wall nitrogen is converted into a non-sedimentable form. These results demonstrate that cell wall N liberation depends upon the proper ionic environment. Presumably the N liberation can occur only after the irreversible step in adsorption which is known to require added ions (2).

*Nature of Material Released from Cell Walls*—The non-sedimentable material from one experiment was dialyzed against distilled water for 48 hours at room temperature. The dialyzable and non-dialyzable fractions were subjected to N partition, and all fractions were assayed for N and for N<sup>15</sup>. 16 per cent of the non-sedimentable N<sup>15</sup> was found in dialyzing water, while 14 per cent remained in the dialysis bag. Although there is an unequal distribution of the material with respect to dialyzability, 50 per cent of both fractions were soluble in 0.3 M trichloroacetic acid. There was no alcohol-soluble N<sup>15</sup> in either the non-dialyzable or the dialyzable fraction, which indicated the absence of phospholipide N in the cell wall material made non-sedimentable.

Weidel cell walls (Preparation 4) were prepared from cells grown in medium containing both N<sup>15</sup>H<sub>4</sub><sup>+</sup> and HP<sup>32</sup>O<sub>4</sub><sup>-</sup>. After these walls were incubated with unlabeled T<sub>2</sub>r<sup>+</sup> bacteriophage (40 T<sub>2</sub> per cell wall), the supernatant solution after centrifugation was assayed for both N<sup>15</sup> and <sup>32</sup>P. It was found that 7.3 per cent of the cell wall N<sup>15</sup> was liberated as

compared to only 3.5 per cent of the cell phosphorus wall P<sub>o</sub>. These results on the nature of the products indicate the complexity of the wall material liberated during interaction with phage.

*Effect of Heat and Phenol on Cell Walls*—Salton and Horne cell walls were heated at 95° for 1 hour in distilled water. Before incubation with bacteriophage the cell walls were centrifuged once and resuspended in water. The results of incubating these heated cell walls with T<sub>2</sub>r<sup>+</sup> at 37° for 30 minutes are shown in Table IV. Heating has no effect on the capacity of the walls to bind bacteriophage and appears to increase the amount of wall N liberated during the interaction with T<sub>2</sub>r<sup>+</sup>.

Salton and Horne cell walls were treated with 1 per cent and 5 per cent

TABLE IV  
*Effect of Heat and Phenol Treatment on Ability of Cell Walls to Release N*

Type of cell walls	Treatment of cell wall	T <sub>2</sub> r <sup>+</sup> adsorbed Cell wall	Cell wall, N released <i>per cent</i>
Salton and Horne	1 Untreated	197	7.2
	2 Heated to 95°	147	16.4
	3 1 per cent phenol	198	20.0
	4 5 " " "	198	0
Weidel	1 Untreated	20	3.9
	2 2 per cent phenol	16	3.5
	3 5 " " "	3.4	2.2

Incubated at 37°, 30 minutes. The initial multiplicity with Salton and Horne cell walls was 200. The initial multiplicity with Weidel cell walls was 20.

phenol by addition of the solid reagent and incubation for 1 hour at 37°. Phenol was removed by washing and centrifuging three times with water. The phenol-treated walls were incubated with T<sub>2</sub>r<sup>+</sup> for 30 minutes at 37°. The results shown in Table IV indicate that treatment with 1 per cent phenol does not remove the adsorption capacity for T<sub>2</sub>r<sup>+</sup> and that it not only does not prevent breakdown of the wall substance during interaction with the phage but also apparently increases the amount of material which can be released. However, treatment with 5 per cent phenol does prevent breakdown of the cell wall upon interaction with the phage, although it does not affect the ability of the treated walls to bind T<sub>2</sub>r<sup>+</sup>. The effect of phenol on Weidel type walls differs from that found with Salton and Horne walls since even 5 per cent phenol did not completely prevent the release of cell wall N, but phenol also decreased the ability of these cell walls to adsorb T<sub>2</sub>r<sup>+</sup> (Table IV).

## DISCUSSION

The nature of one of the reactions occurring after the electrostatic attachment of the virus to the host is the principal problem investigated in these studies. In general our results are in agreement with the general picture of viral invasion described earlier. The release of a constant amount of cell wall material by interaction with virus suggests that an opening or hole must be made in the cell wall for infection to occur. Although our experiments have been exclusively concerned with isolated cell walls, there is every reason to believe that a similar alteration is made in the cell wall of an intact bacterium. Isolated cell walls have a morphology similar to that of intact cells and interact with viruses only under conditions appropriate for adsorption. The kinetics of viral adsorption and number of viral particles the walls can bind are similar to if not identical with those obtained with whole cells. In addition Puck and Lee (22) have found that infected cells are leaky, again suggesting that a change in the cell wall has occurred.

The exact nature of the alteration made in the cell wall by interaction with the virus cannot as yet be determined. At low multiplicities of infection one virus particle caused the release of about 1 per cent of the cell wall N ( $5$  to  $9 \times 10^{-14}$  mg)<sup>2</sup>. This material is obviously heterogeneous in terms of its size and solubility. However, it appears that what are being destroyed are not the viral receptors. Both Jesaitis and Goebel (23) and Weidel and Kellenberger (24) have shown that neither isolated  $T_4$  receptor nor the isolated  $T_2$  receptor is broken down upon interaction with virus. It seems more likely that the portion of the cell wall surrounding the viral receptor and fixing it in its position in the cell wall is destroyed so that receptor material, plus any attached viral tail material, would not interfere with the subsequent injection of the viral DNA into the cell. One might propose, as Weidel *et al.* (20) have done, that the cell wall is composed of various layers of material and that the layer supporting the viral receptor comprises about 15 per cent of total cell wall and is completely destroyed when up to 15 viral particles are adsorbed to the cell or cell wall. Our studies indicate that the breakdown of cell wall material upon interaction with phage has certain properties which are similar to those of an enzymatic reaction. It has been generally believed for some time that bacterial viruses have lytic properties. This early view was strengthened when it was found that, when a large number of bacterial viruses were added to a susceptible cell, the cell was lysed without viral repro-

<sup>2</sup> It is of some interest to note that the mass of the cell wall material released ( $5$  to  $9 \times 10^{-14}$  mg of N) is similar to the mass of the viral DNA ( $5 \times 10^{-14}$  mg of N) which must pass into the cell.

duction occurring (25) (lysis from without) Weidel has assumed that the decomposition of the bacterial cell wall which he observed was the result of the action of an enzyme associated with the bacteriophage Lark and Adams (26) have also suggested that there is an enzyme in the tail of bacteriophage  $T_5$

Puck and Lee (22) have not favored the view that there is an enzyme associated with the bacterial virus They have ascribed the invasion phenomenon involved in bacteriophage infection to participation of a host cell surface enzyme They believe that the attachment process activates an enzyme which is normally "masked" In experiments with phenol-treated cells and cells subjected to moderate heating, they found that no "lysis from without" resulted from interaction of the killed cells with bacteriophage They postulated that the heat or phenol had inactivated the bacterial surface enzyme, blocking the invasion process Our experiments, which show that phenol-treated cell walls and heated cell walls interact with phage and release nitrogenous material as do untreated cell walls, tend to rule out the presence of a cell wall enzyme whose participation is "triggered" by the attachment step Since the bacteriophage is sensitive to inactivation by heat or phenol, it seems more likely that an enzyme on the bacteriophage tail causes the partial breakdown of host cell walls The restriction of movement of the bound enzyme would explain its failure to catalyze the breakdown of all the host cell wall substrate

#### SUMMARY

Isotopically labeled cell walls of *Escherichia coli* have been prepared by two different procedures The preparations contained protein, carbohydrate, and lipide but no nucleic acid Some variations in N content and in physical and biological properties of the various preparations were observed

Bacterial cell wall nitrogen and phosphorus were converted into a non-sedimentable form by interaction with  $T_{2r}$ ,  $T_{2r}^+$ ,  $T_{4r}^+$ ,  $T_5$ , and  $T_{4r}^+$  Protein "ghosts" of  $T_{2r}^+$  were just as active as the intact virus At low multiplicities (up to 15 phages per cell wall) the amount of cell wall material released was proportional to the number of phages adsorbed Each virus released from  $5$  to  $9 \times 10^{-14}$  mg of N, while the maximal amount of N released by large amounts of virus was 15 per cent of the total cell wall nitrogen The material released is composed of a number of components

The release of cell wall N occurs within 2 to 3 minutes at  $37^\circ$  and in the presence of  $0.16$  M NaCl No N is released at either  $4^\circ$  or in the presence of  $0.0003$  M NaCl Prior treatment of cell walls with phenol or heat does not prevent phage adsorption or the subsequent release of cell wall N

It is suggested that an enzyme in the bacteriophage tail alters the cell wall so that the viral DNA may enter the cell

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# THE FORMATION OF HYDROXYASPARTIC ACID FROM DIHYDROXYFUMARIC ACID AND L-GLUTAMIC ACID\*

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It has been reported that dihydroxyfumaric acid is enzymatically decarboxylated to hydroxypyruvate by rabbit muscle preparations (2). In an investigation of this reaction in other tissues as a possible mechanism for hydroxypyruvate and hence serine formation, it was observed that systems consisting of dihydroxyfumarate, L-glutamate, and various tissue preparations gave rise to an unknown ninhydrin-reacting substance. This compound was cleaved by periodate with the liberation of ammonia and had the same  $R_F$  as synthetic hydroxyaspartic acid in a variety of solvents. These results indicated that the unknown compound was hydroxyaspartic acid formed by transamination between oxalloglycolate, the keto form of dihydroxyfumarate, and glutamic acid. In the reverse reaction, hydroxyaspartic acid and  $\alpha$ -ketoglutarate gave rise to glutamic acid, as shown qualitatively by paper chromatography. In this article, certain properties of this system will be discussed and evidence presented for the identification and characterization of hydroxyaspartate as the product of the forward reaction.

## EXPERIMENTAL

*Enzyme Preparation*—A survey of tissues revealed that the enzyme is present in heart, liver, kidney, and brain obtained from a number of animals, sheep brain was selected as the tissue to be used for further studies. Acetone powders of fresh sheep brain were prepared immediately upon procurement of the tissue and were stored at 5° in a vacuum desiccator. The acetone powder was extracted by stirring with 10 volumes of cold distilled water for 20 minutes in the cold room. The insoluble material was removed by centrifugation and the supernatant solution was heated

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† Project Assistant, Wisconsin Alumni Research Foundation.

with constant gentle swirling in a flask immersed in a water bath at 65–67°, until its temperature rose to 65°. It was maintained at this temperature for 3 minutes and then cooled to 0° in an ice-salt bath. The precipitated protein was removed by centrifugation and discarded. The supernatant solution was placed in a beaker immersed in an alcohol-water bath at –8° and stirred until the first ice crystals appeared. Cold (–15°) acetone was then added at a rate such that the drop in temperature paralleled the addition until a final acetone concentration of 32 per cent (v/v) was reached. The precipitated protein was equilibrated with the solution for 30 minutes with stirring at –8°, then centrifuged for 30 minutes at  $2000 \times g$  at the same temperature, and discarded. The supernatant solution was adjusted to a final acetone concentration of 50 per cent (v/v), as described above, in an alcohol-water bath at –20°. After equilibration and centrifugation, the precipitated protein was taken up in cold water and lyophilized. The dried protein was stored in a vacuum desiccator at 5° over alumina and was found to retain its activity for weeks. The fraction was the enzyme preparation used in all the experiments reported in this paper.

*Substrates*—Dihydroxyfumaric acid was obtained from the Aldrich Chemical Company and recrystallized as described by Hartree (3).  $\alpha$ -Ketoglutaric acid was a commercial product.

Hydroxyaspartic acid was synthesized by a modification of the procedure of Dakin (4). The authors are indebted to Dr. William Shive and Dr. C. G. Skinner, Department of Chemistry, University of Texas, for making the details of their method available to them and also for a sample of authentic hydroxyaspartic acid. The synthetic hydroxyaspartic acid used in these experiments was recrystallized from water and hence should be primarily the less soluble racemic isomer designated as "*para*-hydroxyaspartic acid" by Dakin (4). L-Glutamic acid was used as supplied by the manufacturer without further purification.

*Coenzyme*—Pyridoxal phosphate, as the calcium salt, was kindly supplied by Dr. Wayne Umbreit of the Merck Institute for Therapeutic Research.

*Incubation Conditions*—All the experiments were run at 38° in air. Substrates were adjusted to pH 7.0 before use. The enzyme preparation was allowed to equilibrate with the pyridoxal phosphate for 10 minutes at 38° and then the amino acid solution was added. After a second 10 minute equilibration, the  $\alpha$ -keto acid solution was added and the reaction allowed to proceed for 1 hour. The reaction was stopped by the addition of 0.2 ml. of 40 per cent trichloroacetic acid in all experiments except those in which glutamate assays were made. In these cases, the reaction was stopped by heating the reaction mixture in boiling water for 5 minutes.

*Amino Acid Determinations*—Glutamate was assayed by the procedure

of Cammarata and Cohen with the glutamic acid decarboxylase preparation<sup>1</sup> of heat-treated *Escherichia coli*. All the values were collected for carbon dioxide released non-enzymatically under assay conditions. Hydroxyaspartate was determined by measuring the ammonia released after oxidation with periodate. Aliquots of the supernatant solution after deproteinization were transferred to Conway vessels, neutralized, and oxidized with periodate. Liberated ammonia was determined by the micro-diffusion method of Conway and O'Malley (5). As controls, reaction systems containing only the amino acid or the  $\alpha$ -keto acid were used. Blank determinations, without periodate, were made on all supernatant solutions.

*Protein Determinations*—The biuret method was used in the determination of protein concentration.

### RESULTS AND DISCUSSION

In the early stages of this investigation, it was found that dialysis of aqueous acetone powder extracts resulted in a marked reduction in hydroxyaspartic acid formation from dihydroxyfumarate and glutamate. This effect could be reversed by the addition of magnesium ions. Similar results were observed with non-dialyzed enzyme preparations when Versene was added to the reaction mixtures. The magnesium requirement for the formation of hydroxyaspartate from dihydroxyfumarate and glutamate by the partially purified enzyme preparation is shown in Table I. No magnesium requirement could be demonstrated for the reverse reaction by using hydroxyaspartate and  $\alpha$ -ketoglutarate as substrates.

Hydroxypyruvate was inactive in this system. No ammonia liberation after treatment with periodate could be detected with either glutamate or alanine as the amino donor when hydroxypyruvate replaced dihydroxyfumarate (Table II). This ruled out the possible formation of serine from hydroxypyruvate. The latter could presumably arise from oxaloglycolate by decarboxylation.

Evidence that the reaction observed in this system is one of transamination is the amino acid balance study presented in Table III. The data show that in both the forward and the reverse directions the amino acid alone or the  $\alpha$ -keto acid alone was inactive. In the complete systems, however, the appearance of one amino acid was accompanied by an equimolar disappearance of the other amino acid.

At this stage of purification an absolute requirement for pyridoxal phosphate has not been demonstrated. However, this compound was consistently found to stimulate the formation of hydroxyaspartate from dihydroxyfumarate and L-glutamate by approximately 10 per cent.

<sup>1</sup> P S Cammarata and P P Cohen, to be published

TABLE I  
Effect of  $Mg^{++}$  on Hydroxyaspartate Formation from  
Dihydroxyfumarate and L-Glutamate

$Mg^{++}$	Hydroxyaspartic acid
$\mu\text{moles}$	$\mu\text{moles per hr}$
0	0.7
4	4.6
8	5.7
12	6.3
16	7.4
20	7.0

The reaction systems contained 50  $\mu\text{moles}$  of dihydroxyfumarate, 50  $\mu\text{moles}$  of L-glutamate, 30  $\gamma$  of pyridoxal phosphate, and 3.3 mg of enzyme in a total volume of 3.6 ml, 0.01 M  $PO_4$ , pH 7.4

TABLE II  
Hydroxypyruvate Activity with Acetone-Fractionated Enzyme

Reactants	Ammonia (periodate oxidation)
	$\mu\text{moles}$
Dihydroxyfumarate + L-glutamate	8.1
Hydroxypyruvate + L-glutamate	0
" + L-alanine	0

The reaction systems contained 50  $\mu\text{moles}$  of amino acid, 50  $\mu\text{moles}$  of  $\alpha$  keto acid, 10  $\mu\text{moles}$  of  $Mg^{++}$ , 30  $\gamma$  of pyridoxal phosphate, and 4.0 mg of enzyme preparation in a total volume of 3.6 ml, 0.01 M  $PO_4$ , pH 7.4

TABLE III  
Amino Acid Balance Study

The values are given in micromoles

Reactants	Hydroxyaspartate		Glutamate	
	0 time	1 hr	0 time	1 hr
Hydroxyaspartic acid	49.7	50.4	0	0
" " + $\alpha$ -ketoglutarate	50.1	29.5	0	20.2
$\alpha$ -Ketoglutarate	0	0	0	0
Glutamic acid	0	0	47.4	47.3
" " + dihydroxyfumarate	0	7.3	47.9	40.4
Dihydroxyfumarate	0	0	0	0

The reaction systems contained 50  $\mu\text{moles}$  of the amino acid, 50  $\mu\text{moles}$  of  $\alpha$  keto acid, 30  $\gamma$  of pyridoxal phosphate, 10  $\mu\text{moles}$  of  $Mg^{++}$ , and 4.8 mg of enzyme in a total volume of 4.0 ml, 0.01 M  $PO_4$ , pH 7.4

Hydroxyaspartic acid has been isolated from reaction mixtures after the incubation of dihydroxyfumarate and L-glutamate with the enzyme preparation. For isolation purposes, a series of large scale incubations was carried out as described for previous experiments but with a 400-fold increase in all reactants. The reaction mixture was deproteinized with trichloroacetic acid. The supernatant solution was adjusted to pH 7 with Amberlite IRA-400 and passed over a Dowex 50 column in the hydrogen form. The column was washed until free from acid and then eluted with 1 N trichloroacetic acid. The fractions containing hydroxyaspartate were combined and the trichloroacetic acid removed by ether extraction. The solution was then adjusted to pH 7.0 with KOH and then chromatographed on a Dowex 2 formate column. After gradient elution with 1 N formate, the fractions containing hydroxyaspartate were combined and lyophilized. The residue was taken up in a minimal amount of hot water and the solution cooled with resulting crystallization. The compound was recrystallized from water. Yield, 43 per cent of the hydroxyaspartic acid formed in the reaction mixtures.

The isolated compound gave the same  $R_F$  as synthetic hydroxyaspartate in five different solvent systems. Mixtures of the synthetic and isolated compounds gave single spots. The isolated compound gave the following analytical data.

$C_4H_7O_5N$	Calculated	C 32.22, H 4.73, N 9.40, N liberated as $NH_3$ by periodate
		9.40, eq. wt. 74.5
	Found	C 32.36, H 4.72, N 9.37, N liberated as $NH_3$ by periodate
		9.36, eq. wt. 74.5

The equivalent weight was determined by formol titration. The infrared spectra of the isolated compound and of the synthetic hydroxyaspartic acid were identical in every detail.

The isolated hydroxyaspartic acid was optically active  $[\alpha]_D^{25} +51^\circ \pm 2^\circ$ , c, 1.59 in 1 N HCl. Further work is in progress to establish which tartrate is produced from this amino acid by treatment with nitrous acid.

Hydroxyaspartic acid has been shown to inhibit the growth of *E. coli* and *Leuconostoc mesenteroides* (6), and Kun and coworkers have observed the formation of hydroxyaspartic acid in connection with their studies on the tartrate-oxidizing systems of beef heart mitochondria.<sup>2</sup> The physiological role of this amino acid in mammalian systems is at present under investigation.

#### SUMMARY

The formation of hydroxyaspartic acid from dihydroxyfumarate and L-glutamate has been demonstrated. The reaction appears to be one

<sup>2</sup> E. Kun, personal communication.

of transamination between oxaloglycolate and glutamate. The enzymatically produced hydroxyaspartate has been isolated and characterized.

The authors wish to express their gratitude to Miss Marion Bolton and Miss Mary Steiner for valuable technical assistance, and to Dr. Collin Schroeder for the determination of the optical rotation.

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# THE INCORPORATION IN VITRO OF 4-AMINO-5-IMIDAZOLE-CARBOXAMIDE INTO THE POLYNUCLEOTIDES OF PIGEON LIVER CELLS

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4-Amino-5-imidazolecarboxamide- $C^{14}$  (AICA- $C^{14}$ ) was found to be incorporated into the polynucleotide puines and into the allantoin of the rat (1), into hypoxanthine in pigeon liver homogenates (2), and into adenine and guanine from nucleic acids of yeasts (3). Williams and Buchanan (4) isolated a soluble enzyme system from yeast which incorporated the carboxamide- $C^{14}$  into inosinic acid.

The problem of whether or not the carboxamide *per se* is an intermediate in hypoxanthine synthesis has been investigated by Greenberg (5) and by Schulman and Buchanan (6). They demonstrated that the carboxamide itself is not on the path of hypoxanthine synthesis, but that, instead, a derivative of it, the carboxamide ribotide, is probably involved.

Encouraged by these results, the authors have attempted a series of experiments in which the carboxamide- $C^{14}$  was incubated with homogenates, nuclear fractions, and cytoplasmic particles of pigeon liver to determine whether its radioactivity could be incorporated *in vitro* into the polynucleotides of these preparations.

## EXPERIMENTAL

Young pigeons were used as the source of normal livers as well as of the regenerating livers. The lobectomy of livers had been carried out 3 or 4 days previously. After decapitation of the pigeons, the livers were perfused with cold saline. For the preparation of homogenates, 2 gm of liver were treated for 2 minutes with a Teflon pestle homogenizer. Fractionation of subcellular components was carried out by the method of Hogeboom and Schneider (7). A 20 per cent liver homogenate was made from 15 gm of liver suspended in 60 ml of a 0.25 M sucrose solution containing 0.0018 M  $CaCl_2$ . The nuclear fractions were obtained by centrifuging the homogenates three times at  $700 \times g$  for 10 minutes, the mitochondrial frac-

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tion by centrifugation at  $5000 \times g$  for 10 minutes, and the microsomes were sedimented by centrifugation at  $24,000 \times g$  for 60 minutes. Since the time for the final centrifugation was shortened as compared to the instructions given in the original method, the supernatant fraction may contain some small particles. By microscopic examination, a few erythrocytes were found in the nuclear fractions. These fractions were then suspended in 15 ml of the following media, depending upon the type of experiment carried out. The composition of the incubation media is shown as follows.

The incubation media for experiments concerned with the respiration of homogenates and of mitochondria are given in millimoles: potassium phosphate buffer (pH 7.4) 0.105, KCl 1.05,  $MgCl_2$  0.0495,  $\alpha$ -ketoglutarate 0.045, cytochrome *c* 0.0003, adenosine triphosphate, potassium salt 0.015, and Solution F, which consists of carboxamide- $C^{14}$  0.06, ribose-5-phosphate 0.06, sodium formate 0.06, and calcium leucovorin 0.0001. The total volume is 15 ml.

The incubation media for experiments on anaerobic glycolysis of nuclear and of microsomal fractions are given in millimoles: potassium phosphate buffer (pH 7.4) 0.036,  $KHCO_3$  0.375,  $MgCl_2$  0.105, glucose 0.150, potassium pyruvate 0.075, fructose diphosphate, potassium salt 0.030, adenosine triphosphate (ATP), potassium salt 0.00495, diphosphopyridine nucleotide (DPN) 0.0033, nicotinamide 0.60, KF 0.150, and Solution F (see above). The total volume is 15 ml.

The incubation media for the study of aerobic glycolysis of microsomal and of nuclear fractions are given in millimoles: potassium phosphate buffer (pH 7.4) 0.0495,  $KHCO_3$  0.0495,  $MgCl_2$  0.15, fructose diphosphate, potassium salt 0.15, cytochrome *c* 0.0003, ATP, potassium salt 0.015, DPN 0.0033, nicotinamide 0.60, and Solution F (see above). The total volume is 15 ml.

The 4-amino-5-imidazolecarboxamide-4- $C^{14}$  was prepared by one of the authors (T. N.). This compound was chromatographically pure, and its specific activity was 14,800 c.p.m. per  $\mu$ mole. The incubations were carried out at  $37^\circ$  for 100 minutes. For aerobic incubations, the gas phase was oxygen. Thunberg tubes *in vacuo* were used under anaerobic conditions.

The extraction of nucleic acids was carried out by the following procedure. To 15 ml of resuspended liver fractions was added cold concentrated perchloric acid (PCA) to a final concentration of 4 per cent. The solution was clarified by centrifugation, and the residue was washed, with centrifugation, successively with 25 ml of cold 4 per cent PCA with 2.5 mg of non-labeled AICA, cold 3 per cent PCA (repeated four times), cold 1 per cent PCA, cold 1 per cent PCA in 95 per cent EtOH (1:1),

cold 95 per cent EtOH, 95 per cent EtOH-ether (3:1), extracted three times in hot water bath, 10 per cent NaCl, extracted three times in a boiling water bath for 15 minutes, combined supernatant solutions with 4 volumes of 95 per cent EtOH, overnight at  $-5^{\circ}$ , to yield a residue containing the sodium nucleate

When the nucleic acids contained both deoxypentose nucleic acid (DNA) and pentose nucleic acid (PNA), sodium nucleate was digested at  $37^{\circ}$  for 18 hours with 2 ml of 1 N NaOH. The degraded PNA was separated from DNA by acidification of the solution with HCl, followed by the addition of 2.0 volumes of ethanol. The DNA was collected by centrifugation. The supernatant liquid containing PNA was adjusted at pH 8.2 with NaOH. Then, 2.0 volumes of ethanol were added to precipitate PNA. After hydrolysis with 1 N HCl at  $100^{\circ}$  for 1 hour, adenine and guanine were obtained by use of descending paper chromatography as described by Wyatt (8). The spots were detected by an ultraviolet lamp, and adenine was eluted with 0.1 N HCl and guanine with 1 N HCl. The quantities of adenine and guanine were determined by the Beckman type spectrophotometer at 262.5  $m\mu$  for adenine and 249  $m\mu$  for guanine, respectively. The radioactivities were determined in a Q gas flow proportional counter and calculated for samples of infinite thinness. The possibility of contamination of samples of nucleic acids by adsorbed carboxamide- $C^{14}$  was checked by an experiment in which admixture was made of tissue and radioactive substrate with immediate addition of PCA. No radioactivity was observed in the isolated polynucleotide purines.

### Results

Table I demonstrates the results obtained with regenerating pigeon liver homogenate. The partial hepatectomy had been carried out 4 days prior to the experiment. The relative specific activities of the PNA purines were higher than those of the DNA purines. Table II shows the results with nuclear and cytoplasmic fractions of the liver of the same pigeon used for the experiments reported in Table I. It is of interest to point out that the incorporation of carboxamide- $C^{14}$  into DNA was about the same order of magnitude in the homogenate as in the nuclear fraction. As shown in Table II, the relative specific activities of nuclear and cytoplasmic PNA were also in the same order of magnitude.

The results obtained by the incubation of subcellular fractions are demonstrated in Tables III, IV, and V. Practically no radioactivity (Table III) was detected in mitochondrial purines after incubation with radioactive carboxamide. This does not necessarily mean that mitochondria have no capacity at all for biosynthesis of polynucleotide purines, but that some necessary cofactor may be missing. On the other hand, the

microsomes demonstrated a considerable ability to incorporate carboxamide- $C^{14}$  into both purines of nucleic acids

Table IV shows the comparison of the course of the incorporation of carboxamide- $C^{14}$  into microsomal polynucleotides during anaerobic and

TABLE I  
*Incorporation of Carboxamide- $C^{14}$  into Polynucleotide  
Purines in Regenerating Pigeon Liver Homogenate*

	Specific activities		Relative specific activity *	
	Adenine	Guanine	Adenine	Guanine
	<i>c p m per <math>\mu</math>mole</i>	<i>c p m per <math>\mu</math>mole</i>		
PNA	272	179	1 98	1 30
DNA	123	106	0 89	0 77

Specific activity of AICA- $C^{14}$  in the medium = 13,750 c p m per  $\mu$ mole  
 \* Relative specific activity =  $100 \times \frac{\text{molar activity of polynucleotide purine}}{\text{molar activity of AICA in medium}}$

TABLE II  
*Incorporation of Carboxamide- $C^{14}$  into Polynucleotide  
Purines in Regenerating Pigeon Liver Fractions*

	Specific activities		Relative specific activity	
	Adenine	Guanine	Adenine	Guanine
	<i>c p m per <math>\mu</math>mole</i>	<i>c p m per <math>\mu</math>mole</i>		
Cytoplasmic PNA	208	116	1 51	0 84
Nuclear PNA	205	128	1 50	0 93
“ DNA	127	98	0 92	0 71

Specific activity of AICA- $C^{14}$  in the medium = 13,750 c p m per  $\mu$ mole

TABLE III  
*Incorporation of Carboxamide- $C^{14}$  into Cytoplasmic PNA  
Purines in Regenerating Pigeon Liver Fractions*

	Specific activities		Relative specific activity	
	Adenine	Guanine	Adenine	Guanine
	<i>c p m per <math>\mu</math>mole</i>	<i>c p m per <math>\mu</math>mole</i>		
Mitochondria	16	18	0 11	0 12
Microsomes	262	249	1 75	1 66
Supernatant solution with glycolysis	96	57	0 64	0 38
“ “ “ ATP only	4	2	0 027	0 013

Specific activity of AICA- $C^{14}$  in the medium = 15,000 c p m per  $\mu$ mole

aerobic glycolysis The experiment has been carried out with livers of normal pigeons, and the results indicate clearly that during aerobic glycolysis conditions were more favorable for the incorporation of the labeled compound into microsomal purines than during anaerobic glycolysis

The above facts provide strong evidence in support of the hypothesis that the microsomes are the most active site of nucleic acid synthesis in cells Some uptake of labeled compound into the nucleic acids of the

TABLE IV  
*Incorporation of Carboxamide-C<sup>14</sup> into Polynucleotide  
Purines in Normal Microsomes*

Microsomal PNA	Specific activities		Relative specific activity	
	Adenine	Guanine	Adenine	Guanine
	<i>c p m per μmole</i>	<i>c p m per μmole</i>		
Aerobic	25	33	0 24	0 31
Anaerobic	9	16	0 09	0 15

Specific activity of AICA-C<sup>14</sup> in the medium = 10,600 c p m per μmole

TABLE V  
*Incorporation of Carboxamide-C<sup>14</sup> into Polynucleotide  
Purines in Nuclear Fractions*

		Specific activities		Relative specific activity	
		Adenine	Guanine	Adenine	Guanine
		<i>c p m per μmole</i>	<i>c p m per μmole</i>		
With aerobic glycolysis	Normal liver, PNA	95	72	0 65	0 49
	“ “ DNA	62	54	0 42	0 37
	Regenerating liver, PNA	248	138	1 69	0 94
	“ “ DNA	78	73	0 53	0 50
With anaerobic glycolysis	Normal liver, PNA	20	10	0 14	0 07
	“ “ DNA	58	42	0 40	0 29
	Regenerating liver, PNA	24	21	0 16	0 14
	“ “ DNA	52	67	0 35	0 46

Specific activity of AICA-C<sup>14</sup> in the medium = 14,650 c p m per μmole

supernatant fraction occurred during aerobic glycolysis, but this may have been due to the contamination of this fraction with a small amount of microsomes

Since no radioactivity was found in the purines of the supernatant fraction incubated with ATP (Table III) only, it is felt that the incorporation of carboxamide-C<sup>14</sup> into polynucleotide purines must be coupled with some energy-supplying system supplied by respiration or glycolysis

As shown in Table V, the incorporation of labeled carboxamide into DNA purines was observed in regenerating liver cell nuclei as well as in normal liver cell nuclei. Approximately the same amount of radioactivity was recovered from the purines of DNA isolated from samples incubated under conditions of either aerobic or anaerobic glycolysis. However, the purines of nuclear PNA were renewed markedly during aerobic glycolysis, especially in the case of regenerating livers.

#### DISCUSSION

Our preliminary experiments with  $P^{32}$  have shown that the incorporation of the labeled phosphorus into the nucleic acids of subcellular particles required some energy-coupling reactions such as respiration or glycolysis. As demonstrated in Tables II to V, the incorporation of carboxamide  $C^{14}$  into microsomes as well as into nuclei was accompanied by anaerobic or aerobic glycolysis, whereas in the slice or homogenate the source of energy could be supplied by respiration (*cf.* (Table I)).

In regenerating liver cells, nuclear PNA contained the most radioactivity as shown in Table V. In this case, aerobic glycolysis was the most favorable source of energy. This fact seems important from the viewpoint of neoplastic energy metabolism. Since the early work of Warburg (9), it has been known that aerobic glycolysis may be an important way to supply the energy to tumor tissue where the respiration might be restricted.

Although the role of nuclear PNA in metabolism is not clear, many investigators have noted the considerably active nature of nuclear PNA. Grossman and Visser (10) proposed a hypothesis that nuclear PNA might be a precursor of cytoplasmic PNA. This may be true *in situ*. However, according to the results expressed in Tables II and III, the microsomes also have an unusual ability to synthesize cytoplasmic PNA. In these experiments it would seem that cytoplasmic PNA is synthesized in the same order of magnitude as is nuclear PNA.

#### SUMMARY

- 1 The 4-amino-5-imidazolecarboxamide-4- $C^{14}$  was incubated for 100 minutes with pigeon liver homogenate, the nuclear fraction, mitochondria, microsomes, and supernatant solution.

- 2 Incorporation of the labeled compound into polynucleotide adenine and guanine was found in microsomal pentose nucleic acid (PNA) or nuclear PNA and deoxypentose nucleic acid.

- 3 As energy-yielding systems, respiration or anaerobic and aerobic glycolysis was required.

- 4 The site of polynucleotide biosynthesis in cells was discussed.

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# FAT METABOLISM IN HIGHER PLANTS

## VIII SATURATED LONG CHAIN FATTY ACID PEROXIDASE\*

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In a previous communication (1) it was reported that an L- $\alpha$ -hydroxy aliphatic acid was required as a cofactor for the limited oxidation of palmitic acid by a soluble cytoplasmic protein fraction obtained by precipitation with ammonium sulfate from extracts of cotyledons of germinating peanuts. This fraction was designated supernatant enzyme. No additional cofactors were required, and activity was limited to the long chain saturated fatty acids.

This paper will present results of experiments which indicate that a specific long chain saturated fatty acid peroxidase is responsible for the partial oxidation of palmitic acid to carbon dioxide. L- $\alpha$ -Hydroxy aliphatic acid serves as a substrate for trace concentrations of glycolic acid oxidase which, in oxidizing the substrate, generates  $H_2O_2$ .  $H_2O_2$ , in turn, participates in the peroxidation of the long chain saturated fatty acid. Evidence will also be presented which indicates that one of the reaction products is a long chain fatty aldehyde.

### EXPERIMENTAL

*Preparation of Enzymes*—The procedure employed in preparing the fatty acid peroxidase is that described by Castelfranco *et al* (1). We are indebted to Dr. Ross C. Bean of the University of California for a generous supply of glucose oxidase isolated from the red alga *Iridophycus flaccidum*, and rattlesnake venom (*Crotalus adamanteus*), purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, was used as a source of L- $\alpha$ -amino acid oxidase. Glucose dehydrogenase was prepared by the method of Strecker and Koike (2), and horse-radish peroxidase was obtained from Dr. E. E. Conn of this Department.

Luciferase was prepared by the method of McElroy *et al* (3) from freshly harvested cells of *Achromobacter fischeri*, the bacterial luciferase being stored at  $-10^\circ$  for several months without appreciable loss in activity.

\* A report of this work was presented at the Forty-seventh annual meeting of the American Society of Biological Chemists at Atlantic City, April, 1956.

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*Substrates*—Palmitic acid-1- $C^{14}$  was obtained from Tracerlab, Inc., Boston, and other radioactive fatty acids were supplied by Dr I L Charkoff, Department of Physiology, University of California. The Tris<sup>1</sup> salt of DPNH was obtained from Dr E E Conn, and flavin monophosphate was purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio. The ammonium salts of long chain fatty acids were prepared as described previously (1).

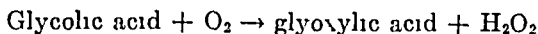
*Methods*—Respiratory  $CO_2$ , derived from the oxidation of labeled substrates by the peroxidase-catalyzed system, was isolated and counted as previously described (1). Long chain fatty aldehydes were assayed qualitatively by the luciferase method developed by McElroy *et al* (3), and light intensity was measured by the Photovolt multiplier photometer, model No 520-M, obtained from the Photovolt Corporation, 95 Madison Avenue, New York 16, New York. Light intensity is recorded as arbitrary light units on the photometric scale of the photometer. In the test procedure, a 0.1 ml aliquot of the reaction mixture is added to the luciferase system containing the following components: 0.5 ml of 0.1 M phosphate buffer at pH 7, 0.05 ml of 1 per cent bovine albumin, 1.4 ml of water, 0.2 ml of  $2 \times 10^{-4}$  M flavin monophosphate, 0.05 ml of luciferase preparation, and 0.1 ml of  $10^{-3}$  M Tris DPNH. The maximal reading on the photometric scale, usually attained in 1 minute, is proportional to the concentration of aldehyde present. The test system is specific for long chain aldehydes; long chain ketones and alcohols are inert.

### Results

*Role of Hydrogen Peroxide*—It has been previously reported that either glycolic acid or one of a series of L- $\alpha$ -hydroxy aliphatic acids was required in the reaction system, since the enzyme catalyzed release of  $C^{14}O_2$  from palmitic acid-1- $C^{14}$  under aerobic conditions (1). The enzyme responsible for this catalysis is found in the supernatant fraction obtained by centrifuging at  $100,000 \times g$  for 1 hour both the mitochondria and the microsomes from homogenates of cotyledons of germinating peanuts. If this extract is then submitted to an acid-ammonium sulfate treatment, as employed by Warburg and Christian to resolve flavoproteins (4), a preparation is obtained which is inactive unless both flavin monophosphate and glycolic acid are added. Since Zelitch and Ochoa (5) have shown that the enzyme responsible for the oxidation of glycolic acid is a flavin monophosphate activated protein, the data gave support to the possibility that glycolic

<sup>1</sup> The following contractions are employed: Tris, tris(hydroxymethyl)amino methane, DPN, diphosphopyridine nucleotide, DPNH, reduced diphosphopyridine nucleotide.

acid oxidase participated in some manner in the oxidation of palmitic acid. Glycolic oxidase catalyzes the following reaction



When an  $\text{H}_2\text{O}_2$ -generating system is added to a reaction mixture of palmitic acid-1- $\text{C}^{14}$  and fatty acid peroxidase, rapid release of  $\text{C}^{14}\text{O}_2$  is observed. No glycolic acid requirement is now demonstrable. As presented in Table I, three completely different types of  $\text{H}_2\text{O}_2$ -generating systems may

TABLE I  
*Activation of Fatty Acid Peroxidase by  $\text{H}_2\text{O}_2$  Generating Systems*

System	C p m as $\text{C}^{14}\text{O}_2$
Supernatant enzyme + L-amino acid oxidase	130
“ “ + “ “ “ + L-leucine	2638
“ “ + glucose dehydrogenase + glucose + M B	117
“ “ + “ “ + “ + “ +	
DPN	1497
Supernatant enzyme + glucose oxidase	120
“ “ + “ “ + glucose	2900

Each Warburg cup contained 0.5 ml of supernatant enzyme (10 mg of protein), 0.1  $\mu\text{mole}$  of palmitic acid-1- $\text{C}^{14}$  (3700 c p m), 50  $\mu\text{moles}$  of potassium phosphate buffer at pH 7.3, and, as indicated, 200  $\gamma$  of L-amino acid oxidase, 10  $\mu\text{moles}$  of L-leucine, 0.1 ml of a liver glucose dehydrogenase preparation, 100  $\mu\text{moles}$  of glucose, 100  $\gamma$  of methylene blue (M B), 0.1  $\mu\text{mole}$  of DPN, and 0.1 ml of a preparation of glucose oxidase (*I. flaccidum*). The total volume in each case was 1.7 ml. 0.2 ml of 20 per cent KOH in the center well and 0.3 ml of 10 N  $\text{H}_2\text{SO}_4$  in the side arm. Time, 1 hour at 25°.

be coupled to the peroxidase which catalyzes the breakdown of palmitic acid. In each case fatty acid peroxidase is an absolute requirement.

The direct addition of reagent  $\text{H}_2\text{O}_2$  to the reaction mixture results in rather low activation. A similar lack of participation by reagent  $\text{H}_2\text{O}_2$  in a peroxidation reaction has been observed with the tryptophan peroxidase (6) and with the catalase-catalyzed oxidation of ethanol (7).

The addition of catalase to the complete system does not result in marked inhibition. The inability of catalase to prevent the action of the peroxidase, the inertness of reagent peroxide in the test system, and the activation by glycolic acid in a system in which the glycolic acid oxidase occurs in low activity would suggest that the peroxidase acts only with enzymatically generated peroxide maintained at a low level, and that the affinity for the formed peroxide must be necessarily high. The peroxidic oxidation of saturated fatty acids is apparently associated with a specific protein. Thus, horse-radish peroxidase in the presence of a peroxide-generating

system, but in the absence of fatty acid peroxidase, does not catalyze the release of  $\text{CO}_2$  from palmitic acid-1- $\text{C}^{14}$

*Inhibitors*—Earlier work showed that the reaction is inhibited by hydroxylamine, imidazole, semicarbazide, and arsenite (1). Further studies now indicate that azide and cyanide are also effective inhibitors. The peroxidase is somewhat insensitive to cyanide since concentrations of  $10^{-3}$  M cyanide gave little inhibition, however, at  $10^{-2}$  M concentration 70 per cent inhibition is observed. Azide, on the other hand, is an effective inhibitor since concentrations as low as  $5 \times 10^{-5}$  M will give 87 per cent inhibition. Although azide has no effect on the peroxide-generating systems, it is a well known inhibitor of peroxidases (8).

*Specificity*—Table II summarizes the activity of the peroxidase toward

TABLE II  
*Specificity of Fatty Acid Peroxidase*

Substrate	Per cent $\text{C}^{14}$ as $\text{C}^{14}\text{O}_2$	Substrate	Per cent $\text{C}^{14}$ as $\text{C}^{14}\text{O}_2$
Capric acid-1- $\text{C}^{14}$	0	Palmitic acid-3- $\text{C}^{14}$	0
Lauric acid-1- $\text{C}^{14}$	<2	“ acid-11- $\text{C}^{14}$	0
Myristic acid-1- $\text{C}^{14}$	46	“ acid-15- $\text{C}^{14}$	0
Palmitic acid-1- $\text{C}^{14}$	50	Stearic acid-1- $\text{C}^{14}$	15
“ acid-2- $\text{C}^{14}$	0		

Each manometer cup contained 0.5 ml of supernatant enzyme (10 mg of protein), 0.1  $\mu\text{mole}$  of the ammonium salt of the suitable fatty acid, 20  $\mu\text{moles}$  of L leucine, 200  $\gamma$  of L-amino acid oxidase, and 50  $\mu\text{moles}$  of potassium phosphate buffer, pH 7.3. The final volume was 1.5 ml. 0.2 ml of 20 per cent KOH in the center well and 0.3 ml of 10 N  $\text{H}_2\text{SO}_4$  in the side arm. Time, 1 hour at  $25^\circ$ .

various substrates. The peroxidase will catalyze only the release of  $\text{CO}_2$  from the carboxyl group of long chain fatty acids but will not participate in a progressive internal oxidation. In addition, oxidation is limited to the long chain fatty acids ( $\text{C}_{14}$ ,  $\text{C}_{16}$ ,  $\text{C}_{18}$ ), the lower homologues are inert. Since isotopically labeled unsaturated fatty acids are unavailable, no tests were carried out for the release of  $\text{C}^{14}\text{O}_2$ .

*Reaction Products*—A solution of the problem of the identity of the reaction intermediates which occur in the oxidation on the long chain fatty acid by the peroxidase is dependent on the development of sensitive methods for their detection. Paper chromatography has not been a useful tool for the preparation of small amounts of long chain fatty acid reaction products. However, it has been possible to demonstrate that long chain fatty aldehydes are produced. McElroy *et al* (3) and Cormier and Strehler (9) have shown recently that luminescence occurs when

DPNH, flavin monophosphate, and a long chain fatty aldehyde are added to an extract of *A. fischeri*. Long chain ketones and alcohols are inert in the *A. fischeri* system. When this system was used as an assay method for the detection of long chain fatty aldehydes, a definite relationship

TABLE III

*Formation of Long Chain Aldehydes As Function of H<sub>2</sub>O<sub>2</sub>-Generating System*

System	Light units
Supernatant enzyme	1,900
" " + glycolic acid	14,000
" " + glucose	1,900
" " + " + glucose oxidase	15,000
" " + glucose oxidase	1,800

Each reaction mixture contained 0.1  $\mu$ mole of ammonium palmitate, 0.5 ml of supernatant enzyme (10 mg of protein), 20  $\mu$ moles of potassium phosphate, pH 7.3, and, as indicated, 10  $\mu$ moles of glycolic acid, 100  $\mu$ moles of glucose, 0.1 ml of a glucose oxidase preparation (*I. flaccidum*). The total volume was 0.8 ml. The reactions were carried out in 10 ml glass-stoppered weighing bottles at 30° on a shaker. After 1 hour, 0.1 ml aliquots were delivered into the luminescent system consisting of 50  $\mu$ moles of phosphate buffer at pH 7.0, 0.5 mg of bovine albumin, 0.04  $\mu$ mole of riboflavin monophosphate, and 0.05 ml of bacterial luciferase. Thereafter, 0.14  $\mu$ mole of DPNH was rapidly added and the maximal light intensity measured by the photomultiplier apparatus.

TABLE IV

*Substrate Specificity for Aldehyde Formation*

Substrate	Light units
Capric acid	0
Lauric "	200
Myristic acid	17,200
Palmitic "	15,000
Stearic "	2,600

The reaction was carried out in essentially the same manner as in Table III. Glycolic acid was employed as the H<sub>2</sub>O<sub>2</sub>-generating system.

between peroxidase activity and long chain aldehyde formation was consistently observed. Thus, in Table III are summarized data which demonstrate the requirement for an H<sub>2</sub>O<sub>2</sub>-generating system in the formation of long chain aldehydes. Furthermore, substrate specificity for long chain aldehyde formation (Table IV) and the release of C<sup>14</sup>O<sub>2</sub> from carboxyl-labeled fatty acids are strikingly parallel. The addition of alcohol dehydrogenase from horse liver to the fatty acid peroxidase system greatly

depresses the ability of this system in stimulating the luminescent activity of the luciferase assay system. This suggests that the long chain aldehydes which are initially formed are being removed by reduction to the corresponding alcohols.

### DISCUSSION

Fatty acid peroxidase may now be added to the group of peroxidases which have well defined substrate specificity. Thus, tryptophan peroxidase and cytochrome *c* peroxidase, unlike the general peroxidases such as horse radish peroxidase, have the unique property of high substrate specificity.

Little is known about the stoichiometry of fatty acid oxidation and aldehyde formation. Thus, other products may well be accumulating which have thus far escaped detection. Since Castelfranco *et al* (1) demonstrated that  $\beta$ -keto palmitic acid is inert, alkyl methyl ketones are probably not produced.  $\alpha$ -Keto palmitic acid is decarboxylated by peanut extracts. However, this substrate does not form significant quantities of aldehydes, indicating that it does not undergo a simple anaerobic decarboxylation. Furthermore,  $\alpha$ -keto palmitate decarboxylation is independent of an  $H_2O_2$ -generating system.

Since the decarboxylation of fatty acids runs parallel to the formation of long chain aldehydes, it is possible that only a straight chain saturated fatty acid is degraded to aldehyde with 1 less carbon atom, which is released as  $CO_2$ . However, at present little information is available to indicate that long chain saturated fatty aldehydes with an odd number of carbons or the corresponding reduction products, the long chain fatty alcohols, accumulate in plant material.

In summary, germinating peanut cotyledons contain at least three mechanisms for the oxidation of long chain saturated fatty acids: (1) the specific fatty acid peroxidase described in this paper, (2) a microsomal system which catalyzes a stepwise degradation of long chain saturated fatty acids (10), and (3) the conventional  $\beta$ -oxidation pathway localized in mitochondrial particles which oxidize both short and long chain fatty acids to acetyl coenzyme A units (11).

### SUMMARY

In extracts of cotyledons of germinating peanut seedlings, there occurs a specific long chain fatty acid peroxidase which peroxidizes stearic, palmitic, and myristic acids with a loss of the carboxyl carbon as  $CO_2$  and the accumulation of a long chain fatty aldehyde. The enzyme is inhibited by cyanide and azide but not by catalase. The role of glycolic acid, previously reported to be required as a cofactor, is that of serving as a substrate for glycolic oxidase which occurs in the enzyme preparations. In

the oxidation of glycolic acid,  $\text{H}_2\text{O}_2$  is formed, which then couples into the peroxidase system

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# PARTIAL DEGRADATION OF THE BENZENE RING OF ESTRONE ISOLATION OF CARBON ATOMS 2 AND 4<sup>\*</sup>

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The biosynthesis of the estrogens from C<sup>14</sup>-labeled acetate has been demonstrated both *in vivo* and *in vitro* (1-5). A determination of the distribution of radioactivity in the labeled estrogens may make possible an elucidation of the mechanisms involved in the biosynthetic pathway from acetate. Although the cleavage of the D ring of the estrogens has been reported (6, 7), the degradation of the benzene ring apparently has not yet been accomplished. This paper describes the application to nitroestrones of the bromopichin "split,"<sup>1</sup> which results in the isolation of carbon atoms 2 and 4 of the benzene ring as bromopichin (tribromonitromethane). For this purpose, the 2-, 4-, and 2,4-dinitroestrones and 2,4-dinitro-17-deoxoestrone were synthesized. A comparison of the melting points of the nitroestrones with the same steroids reported in the literature (11-13) indicates that previous workers had isolated mixtures rather than the pure steroids. From the spectroscopic data reported in this communication, it appears that the previous assignment of the nitro groups in the 2- and 4-nitroestrones should be reversed.

## EXPERIMENTAL

All melting points were taken on a Fisher-Johns melting point block. *2- and 4-Nitroestrones*<sup>2</sup>—The nitrating procedure described by Niederl and Vogel (11) was employed. 100 mg of estrone were dissolved in 3 ml of boiling acetic acid. When the temperature of the solution reached 45°, 47  $\mu$ l of concentrated nitric acid were added slowly with mixing, and the yellow solution was allowed to stand overnight at room temperature.

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<sup>1</sup> This phrase refers to the well known degradation of nitro and polynitrophenols by heating with hypohalite (8, 9). Rafelson *et al* (10) used this reaction to degrade a quinoline derivative.

<sup>2</sup> The 2 nitro and 4-nitro group assignments are the reverse of those made by Niederl and Vogel (11) and Hillmann-Elies *et al* (13). The spectroscopic data presented in this paper support the new assignments.



The yellow crystals of 4-nitroestrone were filtered, washed with acetic acid, and dried. The steroid, after crystallization from aqueous acetic acid, decomposed between 270–280°. Other workers (11, 13) have reported a value of 258° for this steroid.

$C_{18}H_{21}NO_4$  Calculated, C 68.55, H 6.71, N 4.44, found, C 68.49, H 6.86, N 4.41

Treatment of 4-nitroestrone with acetic anhydride and pyridine at room temperature yielded the acetate, which melted at 139.5–140° after crystallization from aqueous methanol.

The acetate was chromatographed on a column of aluminum oxide (Merck, acid-washed) and eluted with benzene. After two crystallizations from aqueous methanol, the colorless steroid melted at 163–165.5°. Hillmann-Elies *et al.* (13) report 166°.

$C_{20}H_{23}NO_5$  Calculated, N 3.92, found, N 4.00

The 4-nitroestrone, isolated after the hydrolysis of the acetate with 2 per cent methanolic potassium hydroxide for 2 hours at room temperature, had the same decomposition point, ultraviolet absorption, and infrared spectra as the nitrosteroid that crystallized from the nitrating mixture.

The reflux of 4-nitroestrone in absolute alcohol containing methyl iodide and sodium resulted in the formation of 4-nitroestrone methyl ether, m.p. 254.5–257.5° (decomposition), after crystallization from absolute alcohol. Niederl and Vogel found 150°.

$C_{19}H_{23}NO_4$  Calculated, N 4.25, found, N 4.22

The filtrate from the 4-nitroestrone filtration was poured into 30 ml of water, and the yellow precipitate was filtered and dried. A benzene solution of the steroid mixture was placed on a column of 20 gm of Merck aluminum oxide. Elution was carried out with benzene and with benzene containing 1 per cent methylene chloride. Evaporation of the solvents left 2-nitroestrone, which was crystallized from 80 per cent alcohol, m.p. 183.5–184°, reported at 155° (13).

$C_{18}H_{21}NO_4$  Calculated C 68.55, H 6.71, N 4.44  
Found " 68.41, " 6.63, " 4.52

The 2-nitroestrone acetate, m.p. 174.5–176.5° (literature 146° (13)), was prepared as described above for the 4-nitro derivative. The melting point was unchanged after elution of the steroid acetate from a column of aluminum oxide.

$C_{20}H_{23}NO_5$  Calculated, N 3.92, found, N 3.98

The rest of the material (from the mononitration mixture) in the column of aluminum oxide was eluted with methanol, and infrared examination of

this material indicated the presence of 4-nitroestrone and 2,4-dinitroestrone. It seems likely that the steroids precipitated by the other workers (11, 13) by pouring all of the nitrated estrone mixture into water were the 2-, 4-, and 2,4-dinitroestrones. Their crystallization procedures do not seem to have yielded pure mononitroestrones. This may account for the discrepancies in melting points between the nitrosteroids and derivatives isolated in this study and those reported previously.

*2,4-Dinitroestrone*—100  $\mu$ l of concentrated nitric acid were added with stirring to 10 ml of glacial acetic acid, at 45°, containing 200 mg of estrone. The solution, after standing overnight at room temperature, was poured into 200 ml of water, and the mixture was extracted exhaustively with ether. The latter was dried over anhydrous sodium sulfate and concentrated *in vacuo* to dryness. After two crystallizations from 80 per cent alcohol, the residue yielded 2,4-dinitroestrone, m p 185–187° (literature, 275° (12)).

$C_{18}H_{20}N_2O_6$	Calculated	C 59.99, H 5.59, N 7.78
	Found	" 60.08, " 5.64, " 7.65

The dinitroestrone, after chromatography on aluminum oxide and crystallization, showed the same melting point. The acetate melted at 185.5–186° and mixed melting point with 2,4-dinitroestrone was 140–150°. Chromatography on aluminum oxide, followed by crystallization, raised the melting point to 187–188.5°. There was some hydrolysis of the acetate on the aluminum oxide column.

$C_{20}H_{22}N_2O_7$	Calculated, N 6.96, found, N 7.01
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*2,4-Dinitro-17-deoxoestrone*—17-Deoxoestrone, m p 131–133.5°, was prepared by a Wolff-Kishner reduction of estrone. 93 mg were dinitrated as described above. The crude dinitrosteroid was taken up in a mixture of 1 part of benzene with 3 parts of petroleum ether of low boiling point and placed on a 30 gm column of Merck aluminum oxide. The column was developed with 400 ml of 3:1 petroleum ether-benzene and 200 ml of 1:1 petroleum ether-benzene. Elution of the desired steroid was accomplished with 600 ml of benzene and 500 ml of benzene which contained 0.3 per cent methanol. Evaporation of the solvents left an oil which could be crystallized from a methanol-water mixture (20:1). 2,4-Dinitro-17-deoxoestrone, melting at 100–103°, was obtained. Further crystallization gave a product which melted at 103.5–104°.

$C_{18}H_{20}N_2O_6$	Calculated	C 62.41, H 6.40, N 8.09
	Found	" 62.46, " 6.59, " 8.14

*Conversion of 4-Nitroestrone to 2,4-Dinitroestrone*—4-Nitroestrone dissolved in glacial acetic acid was mixed with 1 equivalent of concentrated

nitric acid at 45° After standing overnight at room temperature, the dinitrosteroid was isolated as described above for 2,4-dinitroestrone After crystallization from 80 per cent alcohol, the compound was found to be identical with 2,4-dinitroestrone on the basis of melting point and infrared spectrum

*Preparation of Nitro-3,4-xyleneols*—The 2-nitro-, 6-nitro-, and 2,6-dinitro-3,4-xyleneols were prepared by the procedures described by Holler *et al* (14) The melting points were in agreement with those which have been reported

*7-Nitro- and 5,7-Dinitro-6-hydroxytetralin*—A mixture of 5- and 6-hydroxytetralins in glacial acetic acid was nitrated with 1 equivalent of concentrated nitric acid at room temperature The resultant mixture of nitro compounds was chromatographed on a column of aluminum oxide The material eluted with 30 per cent benzene in petroleum ether gave 7-nitro-6-hydroxytetralin, m p 88–88.5°, when crystallized from alcohol (literature, 88° (15)) Elution with 75 per cent benzene in petroleum ether resulted in an oil that could not be crystallized Elution with benzene and evaporation of the solvent left 5,7-dinitro-6-hydroxytetralin After crystallization from alcohol, the melting point was 140–142° (literature, 142° (15))

### *Bromopicroin Split of Nitrosteroids*

*Method A Isolation of Both Bromopicroin and Carbon Dioxide from Reaction*—To 0.3 mmole of dinitroestrone or 0.6 mmole of mononitroestrone (small crystals) and 600 mg of calcium oxide, previously heated for 1 hour in a platinum crucible over a Fisher burner, in a 50 ml three-necked flask, were added 3 ml of boiled, distilled water The flask was stoppered and protected by a tube containing lime It was heated on a boiling water bath to permit formation of the calcium salt of the nitrosteroid and was placed in an ice-salt mixture at –5° 250  $\mu$ l of bromine were added with stirring over a 10 minute period, 5 ml of boiled distilled water were added, the contents of the flask were mixed thoroughly, and a downward condenser was attached The outlet was inserted in the mouth of a chilled 5 ml centrifuge tube The flask was heated gently over a free flame, which caused the bromopicroin to be distilled with the water For the cleavage of 2,4-dinitro-17-deoxoestrone, it was necessary to distill with steam or, alternatively, the contents of the flask were brought to a boil, and the bromopicroin was carried over with a gentle stream of nitrogen (9) When the distillate no longer carried over oil droplets of bromopicroin, the distillation was stopped, and the condenser was fitted with a tube containing lime while the contents of the flask were cooled to room temperature One neck of the reaction flask was fitted with a dropping funnel containing 20

ml of 50 per cent sulfuric acid by volume. Another neck was fitted with a gas inlet tube. The funnel and the gas inlet tube were connected in parallel to nitrogen which came from a barium hydroxide scrubber. The condenser was inserted through a 1-hole rubber stopper to the bottom of a 25 ml suction flask containing phenol at 50°. The outlet of the suction flask was connected in series to three 50 ml centrifuge tubes, each containing about 15 ml of barium hydroxide-potassium hydroxide solution (10:1) and 60  $\mu$ l of *n*-octyl alcohol. The acid solution was allowed to run into the reaction flask under nitrogen pressure. After nitrogen was passed through the acidified reaction mixture for 45 minutes, the barium carbonate in the centrifuge tubes was filtered under a nitrogen atmosphere through a previously weighed sintered glass funnel with a  $\frac{1}{4}$  inch-thick asbestos mat. The barium carbonate was washed thoroughly with carbon dioxide-free distilled water and then with methyl alcohol. The sintered funnel was dried at 110° overnight before being weighed. The carbon dioxide from known amounts of sodium carbonate could be determined with an accuracy of  $\pm 3$  per cent when the above procedure was employed (with omission of the calcium hypobromite and the distillation). When the entire procedure was carried out with the omission of the nitrosteroid, between 1 and 2 mg of barium carbonate were obtained.

The 5 ml centrifuge tube containing the aqueous distillate and the bromopicroin was centrifuged, and the aqueous layer was siphoned off. The bromopicroin was washed twice with 3 ml of water, followed by centrifugation each time. It was transferred with 300  $\mu$ l of methylene chloride to a small test tube. The methylene chloride was dried over anhydrous sodium sulfate, filtered through a small sintered glass funnel into another previously weighed test tube, and evaporated at 65°. The open tube, after standing 2 hours at room temperature, was weighed again. This method of isolation, when applied to 6.7 to 77.3 mg of bromopicroin, resulted in a loss of  $3.6 \pm 1.4$  mg. A sample of bromopicroin from the cleavage of 2,4-dinitroestrone isolated in this manner was subjected to infrared analysis, and the absorption spectrum agreed with that of pure bromopicroin (16).

*Method B Isolation of Bromopicroin*—This procedure is a modification of that described by Baddiley *et al.* (17). To 0.15 mmole of dinitroestrone or 0.3 mmole of mononitroestrone in a 70 ml round bottomed flask were added 600 mg of calcium oxide and 3 ml of distilled water. The mixture was heated in a boiling water bath and then chilled to 0°, after which 250  $\mu$ l of bromine were added with stirring. In a 250 ml beaker were placed 27 ml of distilled water and 5.1 gm of calcium oxide. The mixture was stirred by means of a magnetic stirrer and chilled to 0° while 1.8 ml of bromine were added. The mixture set to a solid paste which was trans-

ferred to the flask containing the nitrosteroid. The contents were mixed thoroughly, and a downward condenser was attached to the flask, which was then heated with a free flame until no more bromopichrin distilled over with the water. The bromopichrin was isolated as described above under Method A, and infrared analysis disclosed the presence of a minor component, possibly dinitiotetrabromoethane.

The infrared data were obtained with the use of a Perkin-Elmer model 21 spectrometer and potassium bromide disks. A Beckman model DU spectrophotometer was employed for the ultraviolet measurements.

## RESULTS AND DISCUSSION

The bromopichrin split of the nitrosteroids can be of value in elucidating biosynthetic pathways for the estrogens only if the positions of the nitro groups are established. The data in Tables I and II support the tentative assignments given in this paper and are at variance with assignments made previously (11, 13). Table I shows that in the 280  $\mu$  region 2-nitro-3,4-xyleneol has a lower absorption maximum and extinction coefficient than the 6-nitro derivative. This may be attributed to steric inhibition of the 2-nitro group resonance with the benzene ring (18). The same explanation was offered by Holler *et al.* (14) to account for the data in the 400  $\mu$  region for the sodium salts of these compounds (Table II). These phenomena also seem to apply to the 2- and 4-nitroestrones. A Fisher-Taylor-Hirschfelder model of 4-nitroestrone revealed that the methylene group adjacent to the nitro group prevented full coplanarity of the latter with the benzene ring. The agreement in absorption maxima and extinction coefficients shown in Table I for 2-nitroestrone, 2,4-dinitroestrone and their corresponding model compounds is close, although the absorption spectra of 4-nitroestrone and its model compound do not coincide. However, it was converted to 2,4-dinitroestrone, identical in every respect with the same steroid prepared by the dinitration of estrone.

Compounds of similar structure frequently exhibit close agreement in their infrared absorption bands because of the presence of such groups as nitro and aromatic C—H, for example. There should be closer agreement in the absorption spectra (fingerprint region) of each nitrosteroid and its model compounds than among the various nitrosteroids. The data in Fig. 1 indicate that this is the case and offer further evidence for the nitro group assignments made from the ultraviolet absorption data in Tables I and II. The four dinitro compounds show strong absorption bands at 744 to 745  $\text{cm}^{-1}$ , 763 to 766  $\text{cm}^{-1}$ , and 902 to 909  $\text{cm}^{-1}$ . 2-Nitroestrone and its model compounds have absorption bands at 760 to 762  $\text{cm}^{-1}$ , 865 to 866  $\text{cm}^{-1}$ , and 886 to 889  $\text{cm}^{-1}$ . The 865  $\text{cm}^{-1}$  band falls in the region of the aromatic C—H deformation vibration expected in a 1,2,4,

5-tetrasubstituted aromatic compound<sup>3</sup> 4-Nitroestrone and 2-nitro-3,4-xenol show bands at 1029 and 1027  $\text{cm}^{-1}$ , respectively, that are absent from all the other compounds studied. Bands at 824  $\text{cm}^{-1}$  in the former substance and at 812  $\text{cm}^{-1}$  in the latter may be caused by aromatic C—H deformation vibrations<sup>3</sup>

The amounts of bromopicroin and carbon dioxide isolated from the partial degradation of the nitrosteroids by Method A are shown in Table III. Since both the 2- and 4-nitroestrones yield bromopicroin, it may be assumed that the bromopicroin resulting from the cleavage of both dinitrosteroids is derived from carbon atoms 2 and 4. Carbon dioxide was isolated from

TABLE I  
Ultraviolet Absorption Maxima and Molar Extinction Coefficients  
of Nitroestrones and Model Compounds

Compound	Maxima	$\epsilon$	Maxima	$\epsilon$	Maxima	$\epsilon$
	$m\mu$		$m\mu$		$m\mu$	
2 Nitroestrone	293-294	8220	364-366	3710		
7 Nitro-6 hydroxytetralin	293	8170	367-369	3655		
6 Nitro-3,4-xenol*	282-284	9285	357-358	4585		
4 Nitroestrone	278	1720				
2 Nitro 3,4-xenol*	278	5310	360	2780	244	3560
2,4-Dinitroestrone	276	6890	353	3405	430	1050
2,4-Dinitro-17-deoxoestrone	278-279	6760	353-354	3575		
5,7-Dinitro 6-hydroxytetralin	275-276	6495	354-356	3270	433-437	1310
2,6 Dinitro-3,4-xenol	274	6795	350	3605	424	1535

\* Hexane solution. All the others were in absolute alcohol. The data on the xenols are taken from the work of Holler *et al.* (14)

the bromopicroin split of every nitrosteroid. In the case of the dinitrosteroids, part of the carbon dioxide comes undoubtedly from carbon atom 3. However, more carbon dioxide was isolated than can be accounted for

<sup>3</sup> In pentasubstituted and 1,2,4,5-tetrasubstituted aromatic compounds, the C—H deformation vibration has been reported to occur between 876 and 859  $\text{cm}^{-1}$ . In the 1,2,3,4-tetrasubstituted derivatives, the absorption is found around 800 to 829  $\text{cm}^{-1}$  (19, 20). Polynitroaromatic compounds appear to be exceptions to these correlations (21).

Hillmann-Ehes *et al.* (13) found a band of moderate intensity at 864  $\text{cm}^{-1}$  in the spectra of their mononitroestrone acetate, m p 166°. Therefore, they assigned the nitro group to the 2 position in this steroid. This band was not present in the spectra of either the 2- or 4-nitroestrone acetates prepared in this study. The use of a single absorption band (864  $\text{cm}^{-1}$ ) appears to be insufficient evidence upon which to base a nitro group assignment.

from this position alone. The degradation of cyclopentanone with calcium hypobromite gave rise to carbon dioxide. Therefore, some of the carbon dioxide from the degradation of 2,4-dinitroestrone and the mononitroestrones may result from the degradation of ring D. It will be necessary to determine the nature of the degradation products other than

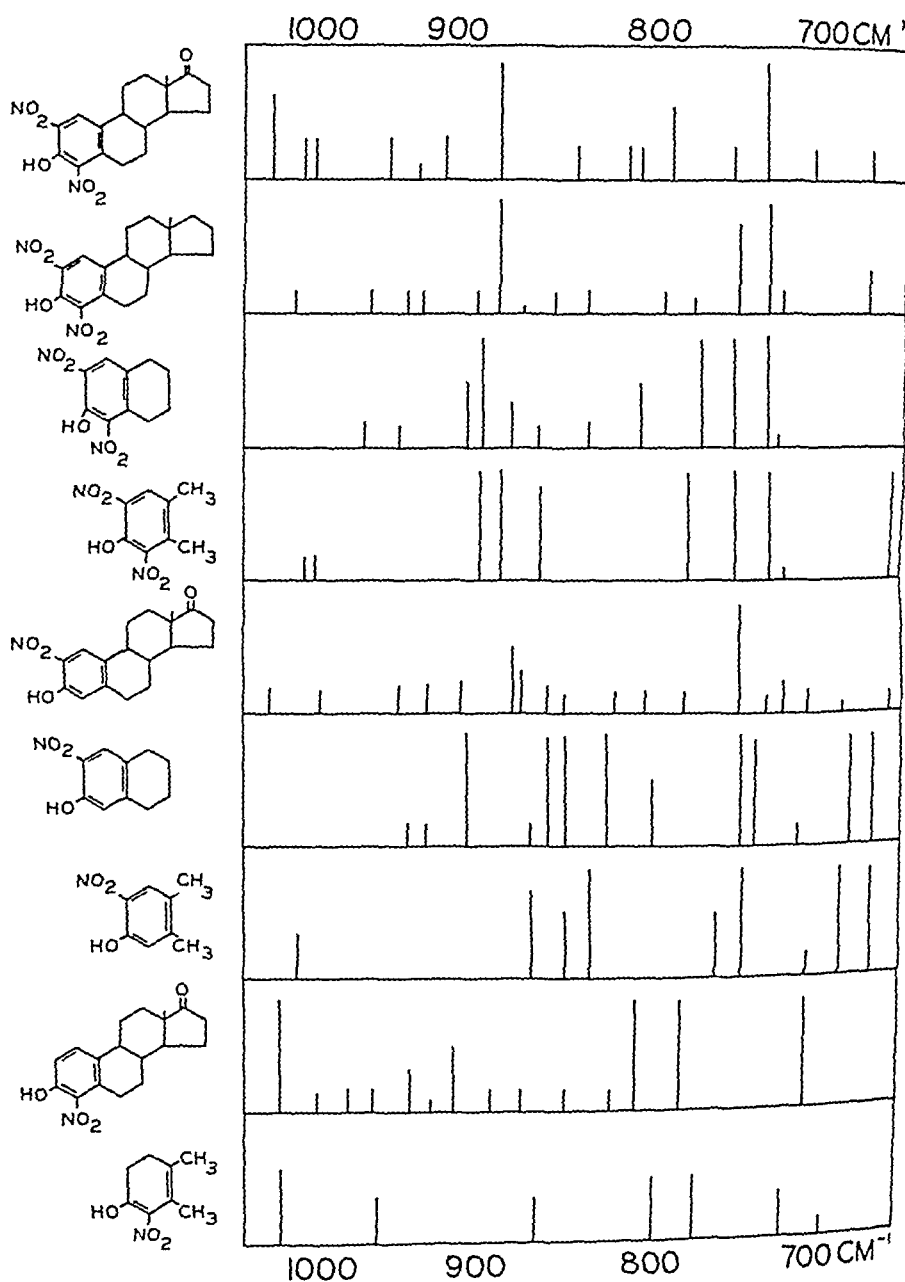


FIG 1 Infrared spectra of nitrosteroids and model compounds in the 700 to 1000  $\text{cm}^{-1}$  region. The lengths of the lines represent relative intensities.

bromopicrin and carbon dioxide before the origin of the latter can be known with certainty

The data in Table III show that, by using Method B for the bromopicrin

TABLE II  
*Absorption Spectra of Sodium Salts of Mononitroestrones and Xylenols*

Compound	Maxima	$\epsilon$
	<i>mμ</i>	
2 Nitroestrone	436-437	4635
4 Nitroestrone	429-431	1031
6 Nitro-3,4-xyleneol*	432	5380
2 Nitro-3,4-xyleneol*	418-420	780

\* The data are taken from Holler *et al* (14)

TABLE III  
*Bromopicrin "Split" of Nitroestrones*

Steroid		Bromopicrin isolated		Carbon dioxide isolated
		Method A*	Method B*	Method A
	<i>mmole</i>	<i>mmole</i>	<i>mmole</i>	<i>mmole</i>
2 Nitroestrone	0 635	0 074		0 35
	0 635	0 060		0 43
	0 322		0 055	
4 Nitroestrone	0 635	0 032		0 25
	0 635	0 057		0 46
	0 338		0 062	
2,4 Dinitroestrone	0 280	0 041		0 42
	0 302	0 16		
	0 305	0 12		0 53
	0 305	0 22		0 51
	0 139		0 095	
2,4 Dinitro-17-deoxoestrone	0 147		0 14	
	0 289	0 055		0 34
	0 289	0 064		

\* Methods of degradation employed as described under "Experimental"

split of the nitrosteroids, about twice as much bromopicrin can be isolated as can be obtained with Method A. However, no attempt was made to measure quantitatively the carbon dioxide arising from the use of Method B since the origin of the carbon dioxide was uncertain. With Method B, the bromopicrin had a minor component not observed in the bromopicrin from Method A.



## SUMMARY

2-Nitro-, 4-nitro, and 2,4-dinitroestrone and 2,4-dinitro-17-deoxoestrone were synthesized. A comparison of the spectroscopic data from the esters with those obtained from model compounds makes it possible tentatively to assign positions to the nitro groups on the benzene ring.

All the nitrosteroids were partially degraded to bromopicrin and carbon dioxide when subjected to the bromopicrin "split". The origin of the carbon dioxide has not been established, but the bromopicrin from the 2 nitro- and 4-nitroestrone is derived from carbon atoms 2 and 4, respectively.

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# OPTICAL CHANGES OCCURRING DURING THE ACTION OF PHOSPHODIESTERASE ON OLIGONUCLEOTIDES DERIVED FROM DEOXYRIBONUCLEIC ACID\*

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Kumitz (2) discovered that the hydrolysis of deoxyribonucleic acid (DNA) by crystalline pancreatic deoxyribonuclease (DNase I) results in an increase of the optical density of the order of 30 per cent. The hydrolysis products of this reaction represent a complex mixture and range in size from mono- to hexa- and possibly to octanucleotides (3, 4). Different opinions have been expressed concerning the spectral effect<sup>1</sup> of the further hydrolysis of some of these fragments. In a recent review, Beaven *et al* (6) quote several papers in which the optical density of an oligonucleotide was accounted for by adding the optical densities of the component mononucleotides. On the other hand, by the same method, Sinsheimer (3) could not account for the optical density of dideoxynucleotides containing guanylic acid.

The availability of the purified phosphodiesterase, which can be used in dilutions having no significant contribution to the ultraviolet spectrum (7), provided a convenient method for the measurement of the optical effect of the cleavage of the internucleotide linkage on several oligonucleotides obtained from the DNA digest. Some of the substrates used were pure dinucleotides of established sequence<sup>2</sup>. The present paper describes the results of this study.

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<sup>1</sup> The term "hyperchromic effect" has been frequently used to describe the increase in optical density during the hydrolysis of nucleic acids (5, 6). The term is not used in the present article because it implies that some standard substance is "normochromic." Usually, nucleic acid itself has been considered as standard substance. Working on the level of dinucleotides, the selection of any one of them as a standard substance does not seem to be justified.

<sup>2</sup> In accord with Smith and Markham (8), and Heppel *et al* (9), the following system of abbreviations is used to describe dinucleotides and their derivatives. The capital letter denotes a nucleoside: A = adenosine, C = cytosine, M = methylcytosine, G = guanosine, T = thymidine, X, Y, and Z denote unspecified nucleosides. The italic *p* denotes a phosphoryl group. If it precedes the capital letter, it signifies 5' phosphate, thus *p*C = 5'-cytidylic acid. If *p* follows the capital letter, it signifies

## EXPERIMENTAL

*Methods*

DNA was prepared according to the method of Kay, Simmons, and Dounce (11) from calf thymus. It was hydrolyzed under the same conditions as previously described (4) for DNase I, which was purchased from the Worthington Biochemical Corporation. The digest was chromatographed<sup>3</sup> on Dowex 1-2X essentially according to Sinsheimer (3) with a few, previously described, modifications (7). Fractions containing dinucleotides were chromatographed a second time, either by using the exact procedure of Sinsheimer (3) or a similar procedure, in which, instead of graded additions of  $\text{NH}_4\text{Cl}$ , the concentration of the eluting formate buffer (7) was stepped up in small increments. In this way several dinucleotides were obtained as the pure compounds of known composition and sequence.

Higher oligonucleotides were also used as substrates for phosphodiesterase, but after one chromatography only, and were designated according to the strength of eluting buffer in which they emerged as "0.5 M fraction," "1.0 M fraction," and "2.0 M fraction" (7).

The method of preparation of phosphodiesterase, units of activity, and conditions of digestion were the same as those previously described (7). The determination of spectral changes was made directly in 1 cm silica cells in the Beckman DU spectrophotometer at 37°. The progress of digestion was also followed by paper chromatography by using the system (isopropanol-water, 70:30, in ammonia atmosphere) of Markham and Smith (12) for nucleotides and the system (*n*-butanol saturated with water in ammonia atmosphere) of Hotchkiss (13) for nucleosides. Some dinucleotides have been dephosphorylated by prostatic phosphatase according to Schmidt *et al* (14).<sup>4</sup>

3'-phosphate, thus Cp is 3'-cytidylic acid. If *p* is between two capital letters as in CpA, it signifies the secondary phosphoryl group linking the 3' carbon of the preceding nucleoside (cytosine) to the 5' carbon of the following one (adenosine). The letter *d* in parentheses signifies that the compound is a derivative of deoxyribonucleic acid, thus (d)pCpA is a deoxyribodinucleotide with a primary phosphoryl in position 5' of cytidylic acid, which is linked from 3' by a secondary phosphoryl group to 5' of adenosine. It has been shown by Sinsheimer (10) that several dinucleotides, emerging from Dowex columns as uniform peaks of constant optical properties, are isomeric mixtures of the type (d)pCpT and (d)pTpC. Usually, one of these forms strongly predominates. The dinucleotides, which are not free from the isomer, are written as (d)[pCpT] to indicate the sequence of the predominant isomer.

<sup>3</sup> We are indebted to the Milwaukee Division of the American Cancer Society for the gift of the automatic recorder for the fraction collector (Gilson Medical Electronics, Madison, Wisconsin).

<sup>4</sup> We are indebted to Dr. G. Schmidt, Dr. P. M. Roll, and Dr. L. Cunningham for the samples of this enzyme.

### Results

The addition of phosphodiesterase to a solution of a pure dinucleotide (d)pCpA results in a progressive increase in the optical density of the solution. Fig 1 illustrates the results of an experiment showing the dependence of the initial rate of the reaction (first 5 minutes) upon the concentration of enzyme. Small amounts of enzyme were used (from  $0.3$  to  $2.0 \times 10^{-3}$  unit) to allow the reaction to proceed sufficiently slowly. The accuracy of the measurements is low, and the scatter of the experimental points is evident from Fig 1. The observed variation might have been caused by instability of the enzyme in the extreme dilutions used.

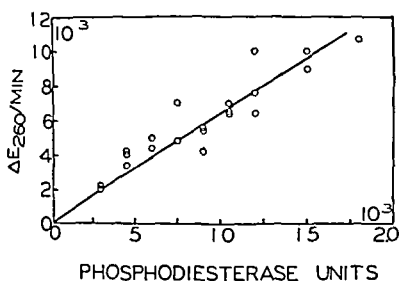


FIG 1

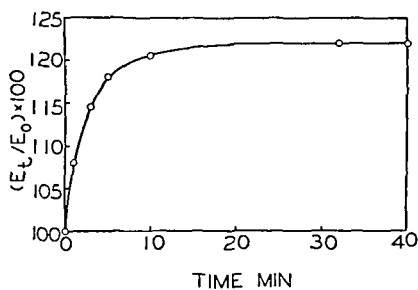


FIG 2

Fig 1 The effect of the concentration of enzyme on the initial rate of the hydrolysis of (d)pCpA. Concentration of substrate adequate to give a reading of slightly over 1.0 at  $260\text{ m}\mu$  at zero time in a Beckman DU spectrophotometer against distilled water. Buffer, glycine-NaOH, pH 9.0, final concentration 0.03 M,  $\text{Mg}^{++}$ , final concentration 0.01 M. The ordinates represent the average increase in absorption at  $260\text{ m}\mu$  per minute during the first 5 minutes.

Fig 2 The optical effect of hydrolysis of "1 M fraction" followed spectrophotometrically. The conditions of hydrolysis were the same as those in Fig 1 except that "1 M fraction" was used instead of (d)pCpA.

In spite of the observed variation, it seems safe to conclude that the initial rate of the reaction is directly proportional to the amount of enzyme. This conclusion is strengthened by the results shown in Fig 6, obtained with the same substrate, indicating that, under favorable conditions, zero order kinetics persist until over 70 per cent of substrate is hydrolyzed.

The experiment shown in Fig 2 was performed with "1 M fraction" which is not a homogeneous substrate. A larger amount of enzyme ( $8 \times 10^{-2}$  unit) was used. The increase in optical density over the original optical density (assumed = 100) is plotted *versus* time. This experiment is cited to illustrate that with a non-homogeneous substrate the kinetics are neither of zero nor of the first order, suggesting that the cleavage of different internucleotide bonds may produce an increase in optical density of a different magnitude, depending on the nature of nucleotides liberated.

In order to establish that the cleavage of the internucleotide bond com-

cides with the increase in the optical density, experiments were performed in which the disappearance of a dinucleotide was measured chromatographically. Fig 3 shows the chromatographic pattern obtained by incubating (d)pCpA with phosphodiesterase. The aliquots were withdrawn at indicated times, placed on the paper, and dried in hot air. The chromatogram was developed according to Markham and Smith (12). The con

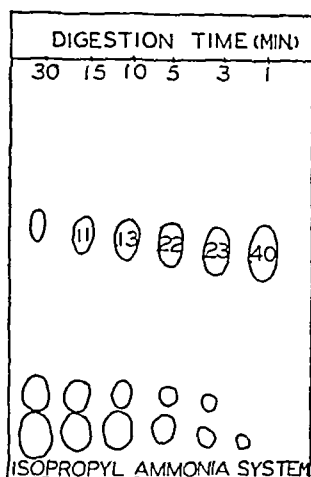


Fig 3

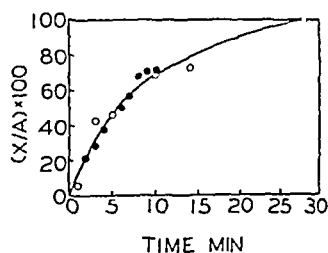


Fig 4

FIG 3 Progress of the digestion of (d)pCpA with phosphodiesterase. 3.2 mg of (d)pCpA (from P determination) in 0.3 ml of  $H_2O$ , buffer, glycine-NaOH, pH 9.0, final concentration 0.1 M,  $Mg^{++}$ , final concentration 0.01 M. After equilibration at  $37^\circ$ , 0.01 unit of phosphodiesterase was added. Aliquots were withdrawn at the indicated times. After chromatography according to Markham and Smith (12), the spots were outlined and eluted. The figures inside each spot represent the values obtained in a Beckman DU spectrophotometer when the eluates were brought to 3 ml and read at  $260 m\mu$ .

FIG 4 Comparison of the rate of hydrolysis of (d)pCpA followed chromatographically and spectrophotometrically. O, data of Fig 3 expressed in per cent of the substrate that disappeared,  $A$  = total amount of dinucleotide and  $X$  = the amount hydrolyzed at time  $t$ . ●, spectrophotometric measurements, in this case  $A$  = maximal increase in the optical density taken as 100 per cent and  $X$  = the increase at  $t$  time.

centration of the unhydrolyzed substrate was determined after eluting the appropriate spots and reading the optical density at  $260 m\mu$ .

The data obtained from this experiment, expressed as per cent of substrate that disappeared, are plotted *versus* time in Fig 4. For the purpose of comparison the data obtained by the spectrophotometric method are also plotted on the same graph. In the latter case the maximal change in optical density (7 per cent of the initial value) is taken as 100, and the change observed at the time indicated is expressed as per cent of the maxi

mal change. With the ratio of enzyme to substrate corresponding to that used in the chromatographic experiment, the agreement between the two methods is satisfactory.

In some experiments it was noticed that, after the first reaction ended, a further incubation for several hours resulted in an additional increase in the optical density. This increase cannot be attributed to the changes occurring as a result of cleavage of the phosphodiester bond, since it was established by chromatography that by that time the hydrolysis had been completed. Furthermore, a corresponding increase in optical density was observed when the mixture of known mononucleotides was incubated for several hours. In all further experiments only the change in spectra

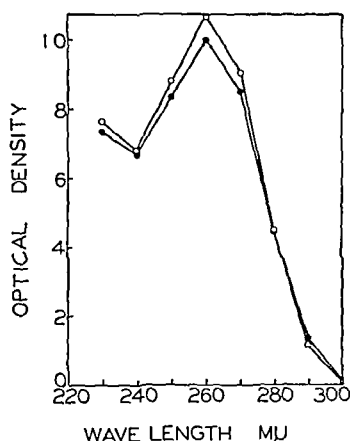


Fig. 5. Spectra of (d)pCpA before (●) and after (○) the hydrolysis by  $8 \times 10^{-3}$  unit of phosphodiesterase for 90 minutes at  $37^\circ$ . Buffer, glycine-NaOH, pH 9.0, final concentration 0.1 M,  $Mg^{++}$ , final concentration 0.01 M.

observed during the period of hydrolysis (not over 4 hours) is considered. An example of the change of spectrum before and after the hydrolysis of (d)pCpA is illustrated in Fig. 5.

In order to establish whether the chemical nature of the nucleotides liberated during the hydrolysis of dinucleotides affects the magnitude of the final optical effect, a number of pure dinucleotides were subjected to the action of phosphodiesterase. The results are summarized in Table I. Unfortunately, not all of the desired combinations were available, and only some of them were free from the contaminating isomer. However, even with those studied it is apparent that the effect is rather small with dinucleotides composed of pyrimidines. The effect is higher with dinucleotides containing a purine. However, with dinucleotides composed of two purines the effect is higher with (d)[pGpA] than with (d)pGpG, whereas only a small difference in the magnitude of the effect was observed

between (d)pGpG and (d)[pTpG], in spite of the fact that the latter contains a pyrimidine

Further experiments were performed on dinucleotides which had first been dephosphorylated by prostatic phosphatase<sup>4</sup> to dinucleoside mono phosphates (XpY). The order of magnitude of the final change in optical density was very similar to that of the original dinucleotide (pXpY)

TABLE I  
*Effect of Complete Hydrolysis of Various Di- and Oligonucleotides by Phosphodiesterase on Optical Density*

Substrate	Per cent increase in optical density at 260 mμ
(d)pCpC	4
(d)[pCpT]	2
(d)pCpA	7
(d)[pTpG]	6
(d)pGpG	7
(d)[pGpA]	11
0.5 M fraction	11
1 M fraction	22
2 " "	33

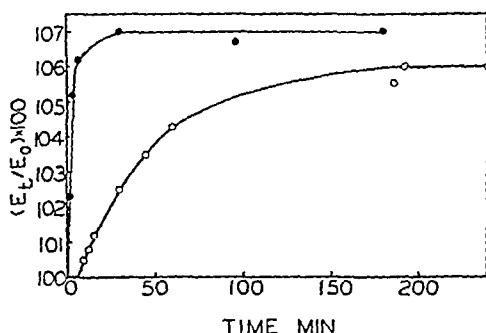


FIG. 6 Comparison of the rates of hydrolysis of (d)pCpA (●) and (d)CpA (○) followed spectrophotometrically. Conditions for both substrates are the same as those in Fig. 1 with the exception that  $4 \times 10^{-2}$  unit of enzyme was used.

However, a striking difference in the rate of the reaction was observed. Two examples are illustrated in Figs. 6 and 7, both were performed with the same pair of substrates, (d)pCpA and (d)CpA, but by the two different techniques. The experiment shown in Fig. 6 was performed by the spectrophotometric method. The lag period with (d)CpA as substrate was observed regularly, but is difficult to explain at present. The final increase in optical density with (d)CpA is almost identical with that of (d)pCpA, but the peak is reached considerably more slowly.

The same general conclusion is drawn from the experiment shown in Fig 7, performed by the chromatographic technique. Since different conditions were used, the rates observed in the experiments of Figs 6 and 7 are not directly comparable. An additional experiment performed by a chromatographic technique on (d)pMpG and (d)MpG gave results almost identical with those shown in Fig 7 and is not reproduced.

Finally, the optical effect of the hydrolysis was studied on fractions composed of mixed oligonucleotides. It has been shown previously (7) that all internucleotide bonds in these fragments are susceptible to phosphodiesterase. Fig 8 illustrates the difference in spectrum of "1 M frac-

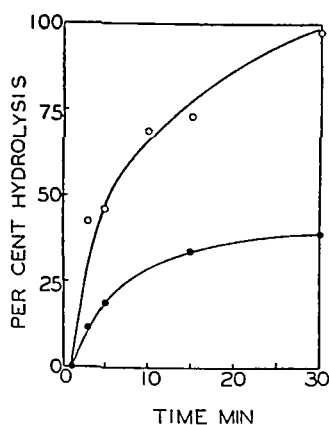


FIG 7

FIG 7 Comparison of the rates of hydrolysis of (d)pCpA (O) and (d)CpA (●) followed by chromatography on paper. Conditions as described in Fig 3, recalculated as in Fig 4.

FIG 8 Spectra of "1 M fraction" before (●) and after (O) hydrolysis by phosphodiesterase. Conditions are the same as those in Fig 5.

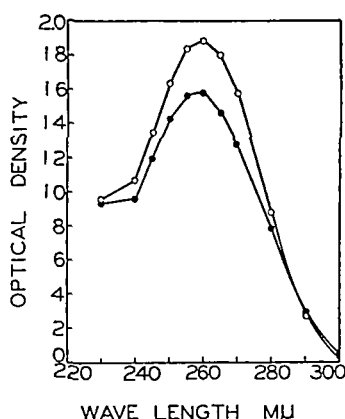


FIG 8

tion" before and after hydrolysis with phosphodiesterase. It might be noted that the largest relative difference is observed not at the height of the peak at 260 m $\mu$  but at 265 m $\mu$ .

The magnitude of the optical effect for various fractions is summarized in Table I. On the basis of chromatographic behavior (3) "0.5 M fraction" is considered to be composed predominantly of trinucleotides, whereas "2 M fraction" reaches probably to hexa- or octanucleotides. The results shown in Table I indicate that the magnitude of the optical effect is correlated with the size of the fragment. In view of the results with various dinucleotides, however, it seems futile to correlate the optical effect with the exact size of the fragment. Yet with the mixed fractions the magnitude of optical effect reflects the approximate size of fragments.



## DISCUSSION

From the results presented in this paper it has been concluded that the cleavage of the internucleotide bond even in dinucleotides and dinucleoside monophosphates results in an increase in optical density. The results further imply that in a compound of the type  $pXpY$  the magnitude of the optical effect depends predominantly on the chemical nature of  $Y$ . The presence of the free phosphoryl group in the 5' position does not seem to affect the magnitude of this effect. It seems premature to speculate on the exact structural rearrangements responsible for the optical effect of cleavage of the internucleotide linkage. Both the base of  $X$  and the base of  $Y$  seem to be involved in the effect, the latter apparently more than the former, in spite of the fact that the free phosphoryl group, which is formed, remains as  $pY$ .

The experiments described also lead to some interesting conclusion and speculations concerning the specificity of phosphodiesterase. Generally, the hydrolysis of all dinucleotides was rapid. Some differences in the rate of hydrolysis were observed when the chemical nature of either  $X$  or  $Y$  was changed, but on the whole these differences were small. On the other hand, the removal of the terminal 5'-phosphoryl group produced a striking decrease in the rate of the reaction. If the enhancing effect of the free 5'-phosphoryl is independent of the length of the chain, the action of phosphodiesterase should resemble that of carboxypeptidase, and the splitting in the compound of the type  $pXpYpZ$  should progress stepwise starting with the liberation of  $pX$ .

Unpublished observations from this laboratory indicate that some deoxyribodinucleotides terminated in 3'-phosphate are hydrolyzed very slowly by phosphodiesterase and require a very large amount of enzyme. These results agree with the previously reported failure (7) to hydrolyze commercial ribonucleic acid (which undoubtedly contains oligonucleotides) by small amounts of purified phosphodiesterase and suggest that the 3' phosphoryl group exerts an inhibiting effect.

## SUMMARY

The cleavage of the internucleotide bond in dinucleotides results in an increase in the optical density. On the whole the effect is small with dinucleotides composed entirely of pyrimidines and large in those containing one or two purines. The optical effect is of the same order of magnitude with a dinucleoside monophosphate as it is with the corresponding dinucleotide. Oligonucleotides larger than dinucleotides show a higher optical effect, which increases with the size of the fragment. Evidence has been presented that the presence of the phosphoryl group in the 5'

position exerts a favorable effect on the action of phosphodiesterase. The specificity of phosphodiesterase has been discussed.

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# CARBON DIOXIDE FIXATION AND UREA SYNTHESIS IN THE RAT\*

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Textbooks and current reviews either make no mention of the fact or leave the impression that the carbon of urea is derived from a general  $\text{CO}_2$  pool. In a study concerned with some aspects of the intermediary metabolism in the irradiated rat, we deemed it essential to establish, first, the part played in glycogen and urea synthesis by the  $\text{CO}_2$  fixation process in the metabolism of glucose and alanine. Our findings were revealing with respect to urea formation, and these important data will be recorded in a separate communication. The form of our experiments and the time and methods of collection were governed by factors not always related to urea synthesis.

## Method

Twenty-four young Sprague-Dawley female rats, 50 to 88 gm, were used in the study. The animals were randomly selected by the cast of a die into groups of six each, *i.e.* Groups A, B, C, and D, which were injected intraperitoneally with  $\text{C}^{14}$ -labeled sodium bicarbonate, randomly labeled glucose (two trials), and alanine labeled in the  $\text{C}^2$  position. After injection, they were placed in metabolism cages (1) designed to separate and collect excreta and to collect expired  $\text{CO}_2$  in towers of  $\text{NaOH}$ . Collections of urine and expired air were made at 1, 2, 3, 4, 5, and 7 hours. At the time of collection the rat was killed, and its liver and muscle glycogen were determined. Thus the value of each time of sacrifice represented the production of one animal biologically integrated over that period of time. Some variations were introduced which probably would not have been present if sequential collections could have been made from one or more

\* Carbon dioxide of plasma, as used in this paper, includes the dissolved  $\text{CO}_2$ ,  $\text{H}_2\text{CO}_3$ ,  $\text{HCO}_3^-$ , and  $\text{CO}_3^{2-}$  as they exist in equilibrium.

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TABLE I  
Percentage of Dose and Specific Activity of  $C^{14}$  in Expired  $CO_2$  and Voided Urine

Group No	After injection	$C^{14}$ expired	Specific activity of carbon expired	$C^{14}$ in urea	Specific activity of carbon in urea*	Specific activity ratio†
		(1)	(2)	(3)	(4)	(5)
Carbon dioxide fixation						
A	hrs	per cent		per cent		
	1	73.87	7028	0.34	333	0.023
	2	74.75	4035	0.61	404	0.101
	3	83.54	3600	0.73	503	0.140
	4	84.42	1930	0.85	455	0.236
	5	86.53	1575	0.89	384	0.244
	7	84.24	1184	0.93	273	0.231
Glucose metabolism (Trial I)						
B	1	5.30	888	0.32	1926	2.169
	2	23.10	1550	1.31	5318	3.431
	3	31.65	1206	1.97	5735	4.700
	4	33.65	1127	1.20	4455	3.913
	5	35.75	975	1.77	4365	1.480
	7	32.45	614	0.57	1865	3.037
Glucose metabolism (Trial II)						
C	1	12.57	1430	0.68	2302	1.610
	2	21.95	1123	3.72	4097	3.648
	3	23.52	979	2.74	4692	4.793
	4	24.00	720	7.90	4318	5.997
	5	27.00	614	6.90	5872	9.563
	7	21.50	403	4.23	4327	10.737
Alanine metabolism						
D	1	4.71	426	0.71	1020	2.391
	2	17.62	957	3.17	3820	3.992
	3	20.95	792	2.46	2920	3.657
	5	25.90	484	1.83	2368	4.892
	7	28.18	404	2.22	2302	5.698

\* Accumulated radioactivity in disintegrations per minute divided by the accumulated mg. of carbon

† Specific activity of carbon in urea divided by the specific activity of expired carbon, i.e., Column 4 divided by Column 2

animals Only one set of values was discarded, that of the 4th hour of the alanine series An adaptation of the method of Dean and Dixon (2) for the determination of an extraneous datum was used in this exclusion

The radioactive materials, with the exception of glucose, were obtained from the Nuclear Instrument and Chemical Corporation of Chicago, Illinois The glucose was extracted by Dr L B Achor from colchicum corms grown by Dr E S Mika in the Department of Pharmacology Biosynthetic Program of the University of Chicago<sup>1</sup> The disintegrations per minute (d p m) per dose administered were sodium bicarbonate 2,825,683, glucose 330,374, and alanine 402,759 In the calculations as presented, all the data are adjusted to a standard dose of 1,000,000 d p m per 100 gm of rat The carrier was as follows glucose 2.85 mg, alanine 4.66 mg, and sodium bicarbonate 2.19 mg

The stable and radioactive carbons were measured as CO<sub>2</sub> by a modified Van Slyke wet combustion method (3)<sup>2</sup> The C<sup>14</sup>O<sub>2</sub> was placed in an ionization chamber (4), the charge in the chamber was then measured with a vibrating reed electrometer,<sup>3</sup> and the disintegrations per minute were calculated by the rate of drift method (5) Urea was determined by the Conway urease microdiffusion method (6) Carbon dioxide was trapped in NaOH and the CO<sub>2</sub> was released, as was that collected from the expired air, from the collecting medium with an excess of phosphoric acid Analyses of C<sup>12</sup> and C<sup>14</sup> were calibrated with benzoic acid of the National Bureau of Standards and C<sup>14</sup> the sodium bicarbonate reference standard, respectively

### Results

The data presented in Table I are given in percentage of the dose administered which appeared in the expired air or urea These data are also tabulated as the specific activity of carbon derived from the expired air and from the urea

### DISCUSSION

We have made two basic assumptions (a) The CO<sub>2</sub> of the expired air is representative of the CO<sub>2</sub> of the pool of plasma and has the same specific activity (7), and (b), similarly, the urea carbon in the voided urine has the same specific activity as that in the body pool of urea from which it was derived In both cases these entities are readily and quickly diffusible

<sup>1</sup> Supported by the Atomic Energy Commission under contract No AT(11-1)-45

<sup>2</sup> A method devised by one of us (L J R) to trap CO<sub>2</sub> with liquid nitrogen for manometric and radiometric measurements To be published

<sup>3</sup> Applied Physics Corporation, Pasadena, California, model No 30

across the lung alveoli and kidney tubule wall and represent an end product of metabolism

If the  $\text{CO}_2$  of the plasma pool serves as the sole precursor for urea, the specific activity of the carbon of urea should eventually equal the specific activity of the carbon of the  $\text{CO}_2$  of expired air. In our study, the carbon fixed as urea from labeled bicarbonate reached an asymptotic level of only about 25 per cent of the carbon in the expired air after 7 hours (Column 5 Table I). Delluva and Wilson (8), after administering radioactive sodium bicarbonate hourly for 18 hours to a rat, found that the ratio was 0.55 at the end of that time. Mackenzie and du Vigneaud (9) fed a rat L-methionine containing  $\text{C}^{14}$  in the methyl group and found the ratio at 24 and 48 hours to be 1.0. In both instances there was essentially a continuous or extended administration of the labeled carbon as carbonate or as a metabolite. It is probable that, because of this, more than just the  $\text{CO}_2$  of the plasma was labeled in their studies, and this could account for the higher ratio values they obtained.

Another surprising fact is that our data show a greater specific activity in the urea than in the plasma  $\text{CO}_2$  after administration of labeled glucose and alanine. Even if labeled plasma  $\text{CO}_2$  had been 100 per cent incorporated into urea, because of the greater specific activity of the urea, one must still conclude that the  $\text{CO}_2$  of the plasma pool is not the sole source of carbon for urea production. Undoubtedly there is another and more significant pathway for  $\text{CO}_2$  fixation as urea. The existing relationship suggests that a large per cent of the urea carbon is derived from a source which is not in ready equilibrium with the pool of plasma  $\text{CO}_2$ . One explanation is that the pool or the source of  $\text{CO}_2$  which finds its way to urea is relatively independent of the pool of plasma  $\text{CO}_2$  because of a different point of origin. It is reasonable to suggest that the liver cells form most of the  $\text{CO}_2$  for the local synthesis of urea, and that other sources form the bulk of the pool of plasma  $\text{CO}_2$  under normal circumstances. There is also the possibility that there exists a direct transcarboxylation or "hand off" mechanism which moves carbon directly to the urea cycle without recourse to the plasma  $\text{CO}_2$  pool. The nature of the mechanism cannot be characterized by this study. The ratios of specific activity of the glucose and alanine trials (Groups B, C, and D) seem to be approximately the reciprocal of the carbon dioxide fixation trial (Group A), and the meaning, if any, of this fact is not immediately apparent.

Dr. Birgit Vennesland of the Department of Biochemistry, The University of Chicago, was consulted frequently. We are grateful for her valuable and pertinent suggestions.

## SUMMARY

1 Carbon fixation as urea can be determined by the ratio of the specific activities of urine urea and the  $\text{CO}_2$  of expired air. At 7 hours this is approximately 25 per cent.

2 After the injection of uniformly labeled glucose- $\text{C}^{14}$  and alanine (labeled in the  $\text{C}^2$  position), the specific activity of urea is higher than that of expired  $\text{CO}_2$ .

3 This fact indicated that urea was formed with the liver cells from plasma carbon dioxide and by some other unknown but more effective mechanism of carbon dioxide production and fixation as urea.

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# STUDIES ON THE MODE OF ACTION OF ANALOGUES OF OROTIC ACID 6-URACILSULFONIC ACID, 6-URACILSULFONAMIDE, AND 6-URACIL METHYL SULFONE\*

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Investigations of the metabolism of orotic acid have shown that this compound can be utilized by certain bacteria (1) and by mammals (2, 3) as a precursor of nucleic acid pyrimidines. More recently it has been established (4-6) that partially purified enzymes from yeast convert orotic acid to U5P<sup>1</sup> in a two-step reaction which involves the enzymes O5P-pyrophosphorylase and O5P-decarboxylase. The presence of O5P-pyrophosphorylase was also demonstrated (6) in the livers of several animal species. Thus, together with other evidence (7, 8), suggests that orotic acid is an important intermediate in the biosynthesis of nucleic acid.

In a previous communication (9), it was shown that 6-uracilsulfonamide and 6-uracil methyl sulfone inhibit non-competitively the growth of two strains of *Lactobacillus bulgaricus* (O9 and Hanson) in media supplemented with orotic acid or with certain precursors of this compound, namely carbamyl-DL-aspartate or L-dihydroorotate. In contrast to the marked effect of this compound on the growth of these organisms, the analogues had but little effect on the growth of *Leuconostoc citrovorum* (*Pediococcus cerevisiae*) 8081 and *Streptococcus faecalis* 8043, organisms which do not utilize orotic acid for growth. It was suggested, therefore, that these analogues exert their toxic effect on certain microorganisms by interfering with one or more stages in the utilization of orotic acid or biologically related compounds.

In the experiments reported here, it has been found that each of the analogues under investigation competitively inhibits the conversion of orotate to O5P by partially purified yeast enzymes. Furthermore, the

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<sup>1</sup> The abbreviations used are uridine-5'-phosphate, U5P, orotidine-5'-phosphate, O5P, 5-phosphoribosylpyrophosphate, PRPP, tris(hydroxymethyl)aminomethane, Tris.

analogues were found to have no effect on the decarboxylation of O5P to U5P

### EXPERIMENTAL

*Synthesis of Labeled Compounds*—Barium cyanamide- $C^{14}$  was prepared from  $BaC^{14}O_3$  by the method of Zbarsky and Fischer (10), thiourea- $C^{14}$  from the crude barium cyanamide by the method of Bills and Ronzio (11), and, from this, orotic acid-2- $C^{14}$  according to the procedure of Heidelberger and Hurlbert (12) Orotic acid-7- $C^{14}$  was prepared<sup>2</sup> according to Langley (13) and also by an improved method (14)

*Synthesis of Analogues*<sup>3</sup>—6-Uracilsulfonic acid was prepared by the method of Greenbaum and Holmes (15) and 6-uracilsulfonamide and 6-uracil methyl sulfone by the method of Greenbaum (16)

*Preparation of O5P-7- $C^{14}$  and O5P-2- $C^{14}$* —These compounds were prepared in essentially the same manner as was O5P by Lieberman *et al* (6) Reaction mixtures (60 ml) containing 80  $\mu$ moles of PRPP and 51  $\mu$ moles of orotate-2- $C^{14}$  ( $1.04 \times 10^7$  c p m) or orotate-7- $C^{14}$  ( $2.19 \times 10^7$  c p m), and 20 units of O5P-pyrophosphorylase (0-0.6  $(NH_4)_2SO_4$  fraction, see "Preparation of enzymes") were incubated at 25° for 1 hour The enzyme was inactivated by heating the solution to 80° for 2 minutes After being cooled and centrifuged, O5P was isolated from the reaction mixtures by chromatography on Dowex 1 (6) 15.1  $\mu$ moles of O5P-7- $C^{14}$  and 17.8  $\mu$ moles of O5P-2- $C^{14}$  were recovered The eluates containing the  $C^{14}$  labeled O5P were neutralized to pH 7.0 with NaOH The O5P was freed of salt by adsorption on small columns of Darco G-60 (2.5 cm  $\times$  1.45 cm) and elution with ammoniacal ethanol The ethanol and excess ammonia were removed by evaporation in a current of air in the cold room

The identity of the compounds was established as follows (1) O5P-7- $C^{14}$  was decarboxylated by O5P-decarboxylase with complete loss of the label (2) U5P resulting from the decarboxylation of O5P-7- $C^{14}$  (specific activity =  $2.02 \times 10^5$  c p m per  $\mu$ mole) had a specific activity of  $2.03 \times 10^5$  c p m per  $\mu$ mole (3) The compound (O5P) showed absorption maxima at 266, 267, and 268 m $\mu$  at pH 7.0, 1.0, and 12.0, respectively The ratio of absorbancies at 280 and 260 m $\mu$  was 0.65, 0.69, and 0.69 at these three pH values, the corresponding values for U5P are 0.37, 0.36, and 0.37 (4) Radioactive homogeneity of the O5P was indicated by chromatography, on Whatman No. 1 paper, with three solvent systems The  $R_f$  values for this compound in *n*-butanol-50 per cent acetic acid (1:1, descending),  $KH_2PO_4$ -isoamyl alcohol (17) (ascending), and isopropanol-formic acid-water (70:10:20, descending) were found to be 0.13, 0.81, and 0.22, respectively, the corresponding values for U5P are 0.22, 0.76, and 0.40

<sup>2</sup> I am indebted to Dr. B. W. Langley for synthesizing this compound

<sup>3</sup> The analogues were synthesized by Dr. S. B. Greenbaum

PRPP was prepared and assayed by the method<sup>4</sup> of Kornberg, Lieberman, and Simms (18). Unlabeled orotic acid, adenosine triphosphate, and ribose-5-phosphate were obtained from the Nutritional Biochemicals Corporation.

*Preparation of Enzymes*—The ethanol fraction containing O5P-pyrophosphorylase and O5P-decarboxylase was prepared from autolysates of dried brewers' or dried bakers' yeast according to Lieberman *et al* (6). It was necessary to incubate some of the brewers' yeast used in this study for periods of 16 to 18 hours at 30° to insure that autolysates would be rich in O5P-decarboxylase.

*Ammonium Sulfate Fractionation*—O5P-pyrophosphorylase and O5P-decarboxylase were separated by  $(\text{NH}_4)_2\text{SO}_4$  fractionation of the ethanol fraction. This fraction prepared from 50 ml of autolysate was dissolved

TABLE I  
*Separation of O5P-Pyrophosphorylase and O5P-Decarboxylase by Ammonium Sulfate Fractionation of Ethanol Fraction*

Enzyme fraction	O5P pyrophosphorylase		O5P-decarboxylase	
	Total units	Specific activity*	Total units	Specific activity
Autolysate	470	0.8	315	0.54
Ethanol fraction	225	1.1	216	0.55
$(\text{NH}_4)_2\text{SO}_4$ , 0-0.6 saturation	50	1.3	0	
" 0.6-0.7 saturation	48	0.47	58	0.32
" 0.7-0.8 "	4	0.03	48	2.1

\* Specific activity = units of enzyme per mg. of protein

in 20 ml of Tris buffer (0.1 M, pH 8.0). The solution was diluted to 40 ml with water and the pH was adjusted to 7.0 by the dropwise addition of HCl (0.1 N), the solution was clarified by centrifugation if necessary. Solid  $(\text{NH}_4)_2\text{SO}_4$  was added slowly, with stirring (temperature 2-4°), to the desired saturation, after being chilled in an ice bath for 30 minutes the precipitate was removed by centrifugation (25,000  $\times g$ , 30 minutes). The ammonium sulfate fractions were dissolved in 10 ml of phosphate buffer (0.05 M, pH 7.5). After dialysis against cold distilled water each fraction was diluted to 20 ml with phosphate buffer. The results of a typical separation are given in Table I. Although a large loss of enzyme results from this fractionation it is seen that the 0-0.6 fraction is devoid of O5P-decarboxylase, the 0.7-0.8 fraction, on the other hand, is rich in the decarboxylase and contains only a trace of O5P-pyrophosphorylase.

<sup>4</sup> The author is grateful to Dr. Arthur Kornberg for having supplied details of this method during the early part of the present investigation through a personal communication to Dr. C. E. Carter.

*Assay of O5P-Pyrophosphorylase*—The spectrophotometric assay of Lieberman *et al* (6) was used in these investigations. In the presence of large amounts of the analogues, however, it was not possible to use this assay. In these experiments orotate was replaced by orotate-7- $C^{14}$ . At the appropriate time, suitable aliquots of the reaction mixture (0.5 ml) were pipetted into 0.1 ml of 10 per cent trichloroacetic acid. The acid mixture was heated in a boiling water bath for 5 minutes to expel the last traces of  $C^{14}O_2$ , after cooling and centrifugation suitable aliquots (usually 0.05 ml) were prepared for counting.

*Assay of O5P-Decarboxylase*—In most of these studies the conversion of O5P to U5P was measured, at 25°, by the loss of  $C^{14}O_2$  resulting from the decarboxylation of O5P-7- $C^{14}$ . The reaction mixture contained O5P-7- $C^{14}$  (0.05  $\mu$ mole,  $1.75 \times 10^4$  c.p.m.), 0.02 ml of  $MgCl_2$  (0.1 M), 0.02 ml of Tris buffer (1 M, pH 8.0), enzyme (about 0.2 unit), and water to volume of 1 ml. The reaction was stopped and the reaction mixtures were treated in the manner described for the assay of O5P-pyrophosphorylase. The spectrophotometric assay of Lieberman *et al* (6) was also used, and the unit of enzyme activity referred to in the present study, for both enzymes, is that defined by these workers.

*Measurement of Radioactivity*— $C^{14}$  activity was measured with a windowless flow counter<sup>5</sup> or with a thin window gas counter<sup>6</sup>. Sufficient number of counts were taken to reduce the statistical error of counting to less than 5 per cent.

### Results

*Effect of 6-Uracilsulfonamide on Conversion of Orotate to O5P*—Early experiments indicated that each of the analogues under investigation inhibited the conversion, by partially purified yeast enzymes (ethanol fraction), of orotate to U5P, as measured by the decarboxylation of orotate-7- $C^{14}$ . The distribution of radioactivity on paper chromatograms of the reaction mixtures from several experiments suggested that the analogues blocked the enzymatic synthesis of O5P from orotate and PRPP, and that they had no effect on the decarboxylation of this compound by O5P decarboxylase.

The effect of 6-uracilsulfonamide on the accumulation of O5P, in the presence of decarboxylase-free O5P-pyrophosphorylase (0.06 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction, Table I), is shown in Fig. 1. These data show clearly that the accumulation of the material represented by Peak A (O5P) is markedly inhibited by this analogue. Although not shown in Fig. 1, the addition of 6-uracil methyl sulfone ( $3.5 \times 10^{-3}$  M) to the reaction mixture caused about a 10 to 15 per cent inhibition of the accumulation of O5P.

<sup>5</sup> Tracerlab SC-16 windowless flow counter

<sup>6</sup> Nuclear D-47 gas flow counter

**Effect of Inhibitor Concentration**—The effect of inhibitor concentration on the rate of conversion of orotate to U5P, by the yeast enzymes, is shown in Figs 2 and 3. A 3-fold excess of O5P-decarboxylase was used in these studies to insure rapid and complete decarboxylation of O5P. It is evident from the Lineweaver-Burk (19) plots that all three analogues competitively inhibit this conversion. The  $K_s$  (dissociation constant) value for orotate,  $2.2 \times 10^{-5}$  M, is in agreement with a previously (6) reported value. The

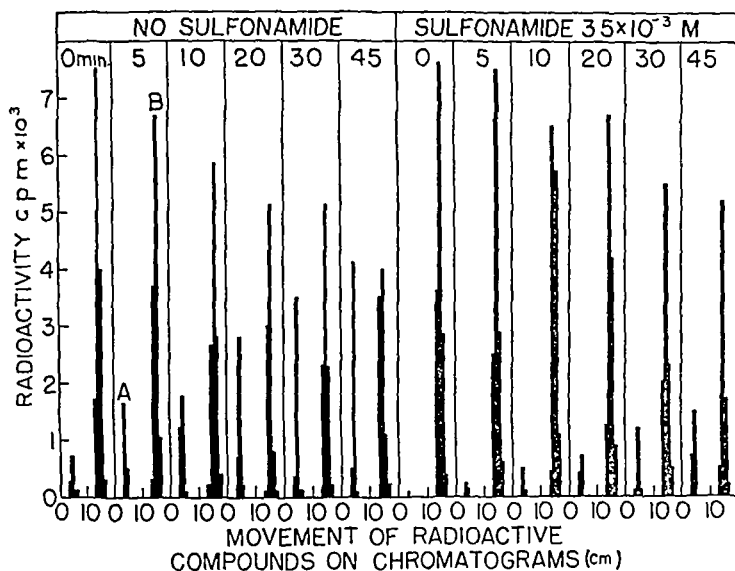


Fig 1 Radioactivity on paper chromatograms. The reaction mixtures (3 ml) contained 0.1 ml of Tris buffer (1 M, pH 8.0), 0.1 ml of  $MgCl_2$  (0.1 M), 0.1 ml of NaF (1 M), 1.92  $\mu$ moles of orotate-7- $C^{14}$  (specific activity =  $8.5 \times 10^5$  c.p.m. per  $\mu$ mole), 2.0  $\mu$ moles of PRPP, 6 uracilsulfonamide (0.026 M, pH 8.0) to desired concentration, and 10 units of O5P-pyrophosphorylase (0-0.6  $(NH_4)_2SO_4$  fraction, Table I). The reaction mixtures were cooled in an ice bath ( $2-4^\circ$ ) prior to adding the cold enzyme. The 0 time sample was removed immediately, the reaction mixtures were then incubated at  $37^\circ$ . At the appropriate time aliquots (0.4 ml) were withdrawn and deproteinized by heating in a boiling water bath for 1 minute. After cooling and centrifugation 0.05 ml aliquots were chromatographed on Whatman No. 1 filter paper (solvent, *n*-butanol-acetic acid-water (50:25:25), descending).

$K_i$  values were calculated to be  $7.0 \times 10^{-5}$ ,  $3.9 \times 10^{-4}$ , and  $7.1 \times 10^{-4}$  M for the sulfonamide, sulfonic acid, and methyl sulfone, respectively.

**Studies with O5P-Decarboxylase**—The time-course of the conversion of O5P-7- $C^{14}$  to U5P, as measured by the disappearance of  $C^{14}$  activity from the reaction mixture, is shown in Fig 4. The rate of the reaction is linear for a period of 12 minutes at  $25^\circ$ . Under the conditions employed in these studies it was found that the rate of decarboxylation of O5P was proportional to the amount of enzyme (Fig 5).

The effect of varying the substrate concentration is shown in Fig 6. The  $K_s$  of the enzyme-substrate complex is  $1.4 \times 10^{-5}$  M O5P.

*Effect of Analogues on O5P-Decarboxylase*—The effect of varying concentrations of the three analogues under investigation on the decarboxylation of O5P was investigated. The analogues were added to reaction mixtures (1.6 ml) containing 0.046  $\mu$ mole of O5P-7- $C^{14}$  ( $1.6 \times 10^4$  c.p.m.) and 0.14 unit of O5P-decarboxylase in concentrations ranging from 2.1

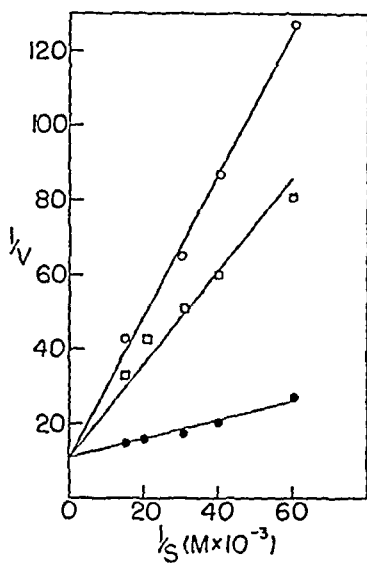


FIG. 2

FIG. 2 The effect of 6-uracilsulfonamide on the rate of conversion of orotate to U5P. The reaction mixture (3 ml) contained 0.05 ml of Tris buffer (1 M, pH 8.0), 0.05 ml of  $MgCl_2$  (0.1 M), 0.40  $\mu$ mole of PRPP, 0.8 unit of O5P-pyrophosphorylase, 2.4 units of O5P-decarboxylase, and varying amounts of orotate. The rate of orotate disappearance was measured by the decrease in optical density during the 0 to 5 minute interval ( $v$ ). ●, varying amounts of orotate, O and □, varying amounts of orotate plus 6-uracilsulfonamide (final concentration  $5.3 \times 10^{-5}$  M and  $2.65 \times 10^{-4}$  M respectively).

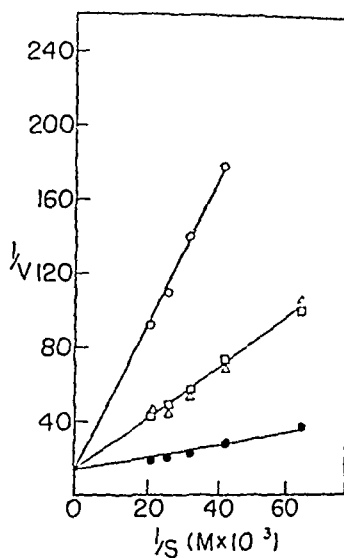


FIG. 3

FIG. 3 The effect of 6-uracil methyl sulfone and of 6-uracilsulfonic acid on the conversion of orotate to U5P. The reaction mixtures were the same as those reported in Fig. 2 except that varying amounts of orotate-7- $C^{14}$  (specific activity =  $8.55 \times 10^4$  c.p.m. per  $\mu$ mole) were used. The rate of orotate disappearance was measured by the decrease in radioactivity, resulting from the decarboxylation of O5P, during the 0 to 5 minute interval ( $v$ ). ●, varying amounts of orotate, □, orotate plus 6-uracil methyl sulfone (final concentration  $2.5 \times 10^{-3}$  M), O and Δ, orotate plus 6-uracilsulfonic acid (final concentration 2.8 and  $1.4 \times 10^{-3}$  M, respectively).

to 12.6  $\mu$ moles per reaction mixture. These concentrations of the analogues had no effect on the rate of decarboxylation of O5P-7- $C^{14}$  during the 0 to 5 minute interval. This, together with the evidence presented in Figs. 1, 2 and 3, suggested that the analogues compete with orotic acid (1) for the active site or sites on the O5P-pyrophosphorylase molecule or (2) for PRPP. In the latter case, the ribotides of the analogues should be formed. Since the analogues, like orotic acid, exhibit a marked shift of the absorption

tion maximum upon changing from acid to alkaline solvent ( $\lambda_{265}$  and  $\lambda_{295}$   $m\mu$ , respectively), ribotide formation would be expected to cause a marked decrease in the optical density of the reaction mixture at 295  $m\mu$ . Accordingly, 6-uracilsulfonamide (absorption maximum 265  $m\mu$  at pH 1.0 and

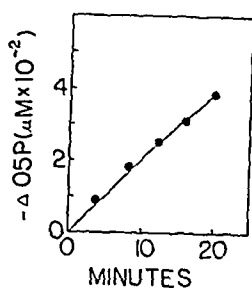


FIG 4

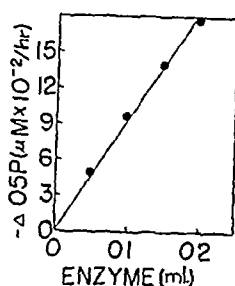


FIG 5

FIG 4 Time-course of decarboxylation of O5P. The reaction mixture (3 ml) prepared as outlined for the standard assay contained 0.26  $\mu$ mole of O5P-7- $C^{14}$  ( $9.1 \times 10^4$  c.p.m.) and 0.4 unit of O5P-decarboxylase. The progress of the reaction was followed by measuring the decrease in  $C^{14}$  activity.

FIG 5 Effect of enzyme concentration on the rate of decarboxylation of O5P. The reaction mixtures (1 ml) (see standard assay) contained 0.093  $\mu$ mole of O5P-7- $C^{14}$  ( $3.2 \times 10^4$  c.p.m.), the enzyme contained 0.9 unit of decarboxylase per ml. Disappearance of O5P calculated from the loss of  $C^{14}$  activity during the 0 to 10 minute interval.

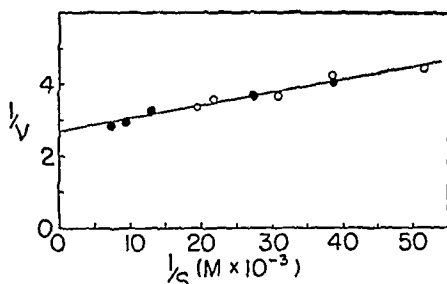


FIG 6 Lineweaver and Burk plot (19) of the rate of O5P decarboxylation as a function of O5P concentration. The reaction mixtures (2 ml) prepared as for the standard assay contained varying amounts of O5P-7- $C^{14}$  (specific activity =  $3.5 \times 10^5$  c.p.m. per  $\mu$ mole) and 0.3 unit of O5P-decarboxylase. The rate of O5P decarboxylation ( $v$  = O5P decarboxylated per hour) was calculated from the disappearance of  $C^{14}$  activity during the 0 to 9 minute interval. O, Experiment I; ●, Experiment II.

295  $m\mu$  at pH 8.0) (0.25  $\mu$ mole) was incubated with PRPP (0.54  $\mu$ mole) and O5P-pyrophosphorylase (0.06  $(NH_4)_2SO_4$  fraction, 1 unit) in a total volume of 3.0 ml. There was no noticeable decrease in the optical density at 295  $m\mu$  during a period of 30 minutes, suggesting that the analogue was not converted to its ribotide.

It might be anticipated that the analogue ribotide, if formed, would



inhibit the decarboxylation of O5P 6-Uracilsulfonamide, in concentrations ranging from 0.82 to 4.1  $\mu$ moles per reaction mixture (2 ml), was incubated for 30 minutes at 25° with PRPP (1.08  $\mu$ moles) and O5P pyrophosphorylase (1.3 units, 0-0.6 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction, Table I). The reaction was stopped by heating in a boiling water bath for 1 minute, the reaction mixture was then immersed in an ice bath. After cooling and centrifugation aliquots of the supernatant solution (0.5 ml) were incubated with O5P-7-C<sup>14</sup> (0.026  $\mu$ mole,  $9.1 \times 10^3$  c.p.m.) and O5P-decarboxylase (0.11 unit). The rate of decarboxylation of O5P, during the interval of 0 to 12 minutes, was not affected by the presence of the first incubation mixture. These findings do not eliminate the possibility of ribotide formation from the analogue, they strongly suggest, however, that, if stable ribotides are formed, they probably exist only in very small amount.

#### DISCUSSION

The results of these studies indicate that the orotic acid analogs 6-uracilsulfonic acid, 6-uracilsulfonamide, and 6-uracil methyl sulfone, competitively inhibit the conversion of orotate to O5P by O5P-pyrophosphorylase. These analogues have no effect on the rate of decarboxylation of O5P by O5P-decarboxylase. These data support the suggestion made in a previous communication (9) that these analogues exert their toxic effect, in part at least, on certain microorganisms by interfering with one or more stages in the utilization of exogenous orotic acid. In view of the present findings, it seems highly probable that one of the major sites of action of the analogues is at the O5P-pyrophosphorylase level. It must be emphasized, however, that inhibition at this site does not suffice to explain the inhibitory effect (9) of these analogues on the growth of *L. bulgaricus* O9.

It was previously reported (9) that, based on the 50 per cent molar inhibition indexes, 6-uracil methyl sulfone was about twice as active as 6-uracilsulfonamide as an inhibitor of the growth of *L. bulgaricus* O9 in a medium supplemented with orotic acid. 6-Uracilsulfonic acid, on the other hand, did not inhibit the growth of this organism. Comparison of the *K<sub>i</sub>* values reported herein shows that the sulfonamide is about 56 times and the sulfonic acid twice as active as the sulfone as an inhibitor of the partially purified O5P-pyrophosphorylase. This difference in activity of the antagonist in the yeast system, *in vitro*, and on bacterial growth may depend on various factors, *e.g.* stability and cellular transport mechanisms or a different order of affinity of the bacterial enzyme for the inhibitors. Since it was found (9) that the inhibition of growth of *L. bulgaricus* O9 by the sulfonamide and the sulfone could not be reversed by orotate or U5P, the possibility of these analogues interfering with some other essential

metabolic process involving one or more, perhaps entirely different, enzymes must also be entertained. That other points in the anabolic processes involving orotic acid are most probably affected by these analogues was suggested by the finding (9) that the effect of these antagonists on the growth of *L. bulgaricus* O9 (1) was not overcome by U5P, (2) occurred also when growth of a mutant strain (O9X) was supported by uracil, and (3) was completely overcome by material, of as yet unknown composition, closely associated with commercially prepared samples of yeast ribonucleic acid. It was suggested that at least one site of action of the analogues lies beyond the mononucleotide stage. The failure to demonstrate a significant formation of ribotides from the analogues by yeast enzymes in this study does not preclude their formation *in vivo* in this or other organisms with subsequent incorporation into polynucleotide fractions. Other pyrimidines, namely 2-thiouracil (20) and 5-bromouracil (21-24), have been shown to be incorporated into nucleic acids. Also, Prusoff, Lajtha, and Welch (24) have presented evidence suggesting that the thymine analogue, 6-azathymine, may be incorporated into a unit more complex than the nucleoside, and, in fact, 6-azathymine has been recovered from the deoxyribonucleic acid of *S. faecalis* grown in the presence of the analogue. The complete elucidation of the mode of action of the sulfur analogues of orotic acid must await the results of further investigation.

#### SUMMARY

1 The orotic acid analogues, 6-uracilsulfonic acid, 6-uracilsulfonamide, and 6-uracil methyl sulfone, competitively inhibited the conversion of orotate to orotidine-5'-phosphate (O5P) by partially purified O5P-pyrophosphorylase of yeast.

2 These analogues had no effect on the conversion of O5P to uridine-5'-phosphate by O5P-decarboxylase.

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# THE SEPARATION AND DETERMINATION OF CYCLIC IMINO ACIDS

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In order to carry out an investigation of the cyclization of  $\delta$ -hydroxylysine to 5-hydroxypipicolinic acid and to study the separation of the diastereoisomers of the latter compound (1), it was necessary to devise a method for the determination of 5-hydroxypipicolinic acid in the effluent from an ion exchange column. None of the methods in the literature which have been used for similar compounds was directly applicable. It was found that Schweet's modification (2) of Chinard's procedure (3), as adapted to pipicolinic acid, was suitable with further modification. The method as finally applied to 5-hydroxypipicolinic acid could also be used for proline, pipicolinic acid, baicalian (4,5-dehydropipicolinic acid), and, with an additional modification, for hydroxyproline. Since the colorimetric procedure was quite general for cyclic imino acids and since any of these compounds, together with a number of others known to exist in nature (4, 5), might be found in an unknown sample, a separation procedure seemed necessary if the method was to be generally useful. This was accomplished by ion exchange chromatography. A somewhat similar procedure for the determination of pipicolinic acid has recently appeared (6). Qualitative identification of the cyclic imino acids, including the diastereoisomers of the hydroxyimino acids, was made by paper chromatography.

## Methods

*Ion Exchange Chromatography*—Dowex 50-X12, minus 400 mesh,<sup>1</sup> was rescreened through a 200 mesh sieve with the aid of a jet of water. The resin was cleaned, and a 50  $\times$  0.9 cm. column was poured as described by Moore and Stein (7). The jacketed column was operated at 50° and mounted on a Technicon fraction collector with a drop-counting attachment. Before each run the column was washed with 50 to 100 ml. of 0.25 N NaOH containing 1 per cent of the detergent solution employed by Moore

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<sup>1</sup> This is a dry size designation. Approximately 90 per cent of the wet resin (H form) should pass a 200 mesh (74  $\mu$ ) sieve. During the screening the sieve openings become clogged. They are most easily freed by drying the sieve for a few minutes in an oven at 100° and then rapping it sharply against a hard surface.

and Stein (7) and then with 25 to 50 ml of citrate buffer at pH 3.10 containing the same amount of detergent. The buffer of pH 3.10 was prepared by dissolving 245 gm of sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ) and 600 gm of citric acid ( $\text{H}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$ ) in water and adding water to make 10 liters. The buffer was kept in the cold with thymol as a preservative. The eluting and washing solutions were introduced into the column through small bore glass tubing set in an 18/9 ball joint clamped to a matching socket on the top of the column. The sample, in a small volume (1 ml or less), of pH less than 3.10, was placed on the top of the resin column and allowed to run in by gravity. This was followed by two small washes, about 0.5 ml each, of the buffer at pH 3.10.

A pH gradient (8) was employed to elute the imino acids. The apparatus consisted of a reservoir and a mixing chamber, both 125 ml. Erlenmeyer flasks, joined near the bottoms of the flasks through glass outlets and Tygon tubing. A second outlet near the bottom of the mixing chamber was connected to the top of the column through 1 mm bore plastic tubing. With the tubing clamped, 125 ml of buffer of pH 3.10 were placed in the mixing chamber and 125 ml of 0.25 N NaOH were placed in the reservoir. Both solutions contained detergent as before. The tubing between the flasks was filled with alkali, and the tubing to be connected to the column was filled with buffer. The flasks were set side by side at the same level with the mixing chamber centered over a magnetic stirrer to provide continuous stirring. The levels were adjusted by adding a small amount of alkali so that the level in the reservoir was a few mm above the level in the mixing chamber to compensate for the higher density of the buffer. The experimental arrangement provided a concave pH gradient and a constant sodium ion concentration (8).

When the sample had been washed into the column, a few ml of buffer of pH 3.10 were placed on the top of the column, and the tubing from the mixing chamber was connected to the top of the column. The inlet tube was tilted so that entering buffer would run down the side. The clamps were removed and elution was allowed to proceed at 5 to 6 ml per hour. To achieve this rate it was necessary to locate the mixing chamber about 60 cm above the top of the column. Alternatively, pressure could be applied to both flasks of the mixing device. The column was used without repouring for all of the experiments with no change in efficiency.

1 ml fractions were collected in 18 × 150 mm test tubes which had been matched as described by Moore and Stein (9). The samples were analyzed directly in these tubes.

*Colorimetric Procedure A*—To 1 ml fractions from the ion exchange column were added 7 ml of freshly prepared 0.15 per cent ninhydrin in

glacial acetic acid This reagent was added with an automatic pipette<sup>3</sup> with sufficient force to provide mixing For the determination of hydroxyproline the tubes were kept at room temperature (24°) for 130 to 140 minutes and then read in a Beckman model B spectrophotometer at 350 m $\mu$  against water Allohydroxyproline gave maximal color in less time, 100 minutes The average absorbance of blank tubes from the column was subtracted from each reading, and concentrations were determined from a calibration curve, prepared from analyses of known amounts of the imino acid in the buffer at pH 3.10 carried through the same procedure In some instances proline was determined in the same manner, except that 280 minutes were necessary for the tubes to reach maximal color

*Colorimetric Procedure B*<sup>4</sup>—As in Procedure A, 7 ml of 0.15 per cent ninhydrin in glacial acetic acid were added to the fractions from the column The tubes were covered with aluminum caps and placed in a vigorously boiling water bath for 35 minutes They were then removed, wiped dry, and allowed to cool to room temperature The tubes in the region of 5-hydroxyisoleucic acid were read in a Beckman model B spectrophotometer at 350 m $\mu$ , proline at 510 m $\mu$ , bakiam at 395 m $\mu$ , and isoleucic acid at 565 m $\mu$ , all against water Absorbances from blank portions of the effluent were averaged and subtracted from each reading Approximate concentrations were determined from calibration curves It was necessary to carry standards<sup>5</sup> through the procedure at the same time as the samples for good precision

*Paper Chromatography*—The methods used were those of Irreverre and Martin (11) as applied to Schleicher and Schuell No. 598 filter paper with the order of the solvents reversed The following details were adhered to In the first dimension 35 ml of *tert*-butyl alcohol-formic acid (88 per cent)-water (70:15:15 by volume) were employed The flow rate was controlled by using a four strand wick to carry the solvent from the reservoir to the top of the filter paper, a distance of 2.8 cm The descension was complete in about 18 hours In the second dimension 80 ml of *tert*-amyl alcohol-2,4-lutidine-water (178:178:114 by volume) were employed The flow rate was adjusted by using sixteen double strand wicks set in a circle 6.8 cm in diameter with the center cut out to facilitate handling The solvent

<sup>3</sup> Cornwall continuous pipetting outfit, Becton, Dickinson and Company, Rutherford, New Jersey

<sup>4</sup> In the original procedure (1) 1 ml of 2 per cent ninhydrin in glacial acetic acid was employed, followed by dilution after color development The newer modification gives similar results and avoids the dilution

<sup>5</sup> The 5-hydroxyisoleucic acid used in early work was kindly supplied by Dr. A. I. Virtanen In later work a sample isolated from dates by a method similar to that described by Virtanen and Kari (10) was employed The isoleucic acid was a gift from Dr. F. C. Steward The bakiam was obtained from Dr. F. E. King

level was set 2.6 cm from the top of the filter paper. Development was complete in 22 to 24 hours but could be stopped in 18 hours with nearly as good results.

### RESULTS AND DISCUSSION

**Color Development**—Procedure B was designed primarily for the determination of 5-hydroxyproline. Although the conditions are not necessarily optimal for the other cyclic imino acids, the method can be used for proline, bialkian, and pipecolic acid. Procedure A is suitable for hydroxyproline, allohydroxyproline, and proline.

In neither procedure is the volume of ninhydrin reagent or the concentration of ninhydrin critical as long as the same conditions are reproduced.

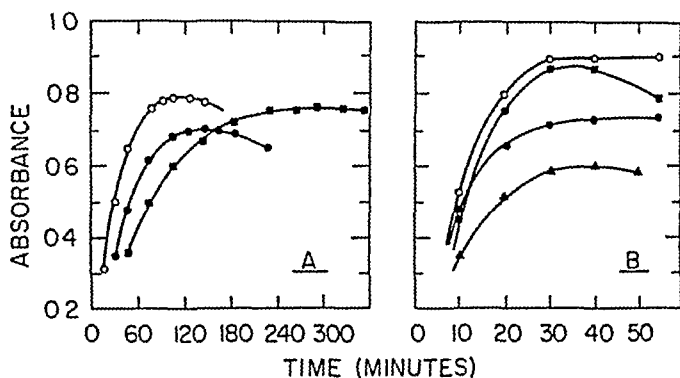


FIG. 1. A, color development *versus* time. Procedure A, ●, hydroxyproline (0.391  $\mu$ mole), ○, allohydroxyproline (0.377  $\mu$ mole), ■, proline (0.401  $\mu$ mole). B, color development *versus* time. Procedure B, ●, 5-hydroxyproline (0.209  $\mu$ mole), ○, proline (0.249  $\mu$ mole), ■, pipecolic acid (0.252  $\mu$ mole), ▲, bialkian (0.191  $\mu$ mole).

each time. The volume chosen, 7 ml, was a convenient one for the 18 X 150 mm spectrophotometer tubes. The concentration selected, 0.15 per cent, was a compromise between better sensitivity at higher ninhydrin concentrations and the necessity of keeping the absorbance of the blanks at a reasonable level.

The rate of formation of color derived from hydroxyproline, allohydroxyproline, and proline (Procedure A) is presented in Fig. 1, A. The color from allohydroxyproline reaches a maximum in about 100 minutes and does not change markedly for about 15 minutes. The absorbance from hydroxyproline is highest after about 130 minutes. Proline is much slower, reaching a fairly broad plateau in about 280 minutes. The effect of different times in the water bath (Procedure B) on the color development of 5-hydroxyproline, proline, bialkian, and pipecolic acid appears in Fig. 1, B. In all cases maximal color is reached in 35 minutes. The colors are stable at room temperature for at least several hours. In the case of

proline and 5-hydroxyproline, they are even stable in the boiling water bath. The pH of the citrate buffer has no influence on the color in either procedure in the range 3.10 to 4.00. Other buffers or buffers of different strengths were not tried, nor was the effect of temperature investigated.

The nature of the colored compounds was not investigated. Troll and Lindsley (12) have suggested that the acid condensation product of proline and ninhydrin is the enol form of the di-(diketohydrindilidene)pyrrole. It is probable that the other cyclic imino acids, except the hydroxyprolines,

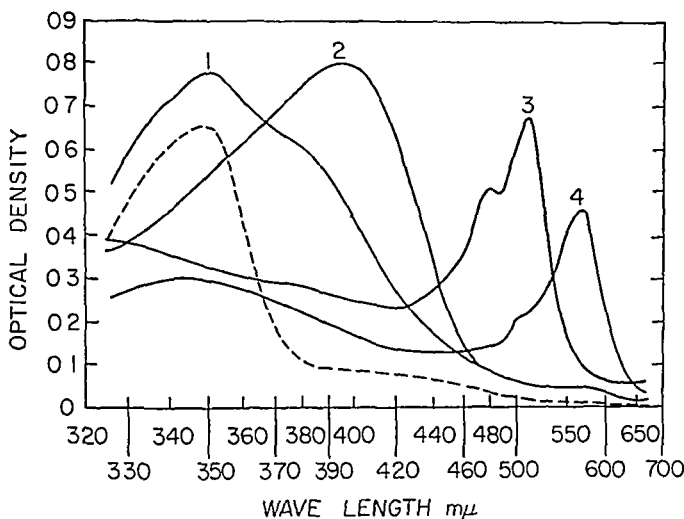


Fig. 2. Absorption spectra of the cyclic imino acids after reaction with ninhydrin in glacial acetic acid. The dash line was obtained with proline (0.401  $\mu$ mole) by Procedure A; hydroxyproline and allohydroxyproline gave the same spectrum. The solid lines were obtained by Procedure B: Curve 1, 5-hydroxyproline (0.324  $\mu$ mole); Curve 2, baicalin (0.382  $\mu$ mole); Curve 3, proline (0.401  $\mu$ mole); Curve 4, pipercolic acid (0.383  $\mu$ mole). The spectra were taken on a Beckman DK 2 recording spectrophotometer with 1 cm. cells.

form analogous compounds in Procedure B. However, this cannot be a complete explanation, since the amount of color obtained by Procedure B is not reproducible. The product obtained from proline and hydroxyproline at room temperature (Procedure A) may represent the condensation of 1 molecule of ninhydrin with 1 molecule of imino acid.

**Absorption Spectra.**—The absorption spectra of the cyclic imino acids appear in Fig. 2. Hydroxyproline, allohydroxyproline, and proline give identical absorption spectra by Procedure A with a maximum at 350 m $\mu$ . The other cyclic imino acids studied show no light absorbance under the conditions of Procedure A in amounts less than 0.5  $\mu$ mole. In Procedure B, 5-hydroxyproline, proline, baicalin, and pipercolic acid give en-



tirely different absorption spectra in spite of the similarity of structure. The absorption maxima appear at 350, 510, 395, and 565  $m\mu$ , respectively. By this procedure, hydroxyproline and allohydroxyproline show a small absorbance throughout the visible region with a plateau around 350  $m\mu$ , but the color yield at this wave length is only about one-tenth that produced by 5-hydroxypipicolic acid.

**Calibration Curves**—A calibration curve for hydroxyproline (Procedure A) appears in Fig 3 which is linear to an absorbance of about 0.5 and then curves slightly. Each point is the average of five single determinations done on different days. The standard deviation around these points is

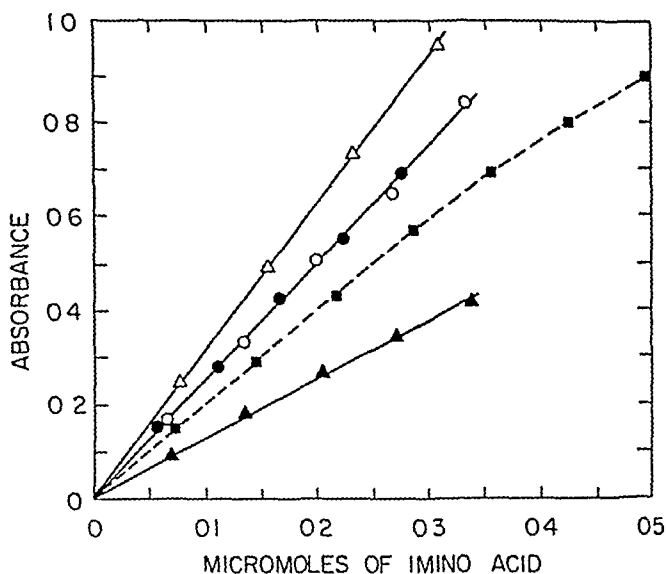


FIG 3 Calibration curves. The dash line represents hydroxyproline analyzed by Procedure A. The solid lines are typical curves determined by Procedure B: ●, 5-hydroxypipicolic acid, ○, proline, ▲, pipecolic acid, △, bairian. A Beckman model B spectrophotometer and 18 × 150 mm test tubes were used.

0.010 absorbance unit, which is equivalent to 0.005  $\mu$ mole of hydroxyproline in the linear portion of the curve. Although allohydroxyproline and proline have not been examined as thoroughly, they appear to show a similar degree of reproducibility but somewhat different color yields. By a single determination the slope of the proline calibration curve was 4 per cent greater than the hydroxyproline curve, while the slope of the allohydroxyproline curve was 15 per cent greater.

Unlike Procedure A, Procedure B does not give reproducible results from one day to the next. Typical calibration curves are shown in Fig 3. The slopes observed for 5-hydroxypipicolic acid, proline, and bairian varied between about 2.5 and 3.5. Pipecolic acid usually had a slope near 1.5 but on occasion was as high as 3.0. However, as is apparent from Fig 3,

the precision within a single group of determinations is excellent, and the calibration curves are essentially linear, accurate determinations can be made by including standards with the unknowns

**Ion Exchange Chromatography**—The separation of a mixture of cyclic imino acids by ion exchange chromatography is demonstrated in Fig 4 With the 50 cm column the separations are complete in every case For many applications a considerably shorter column could be employed For example, hydroxyproline and proline can be separated easily on a 15 × 0.9 cm column of Dowex 50 by using the buffer at pH 3.10 without a gradient A similar procedure has been described by Rogers *et al* (13)

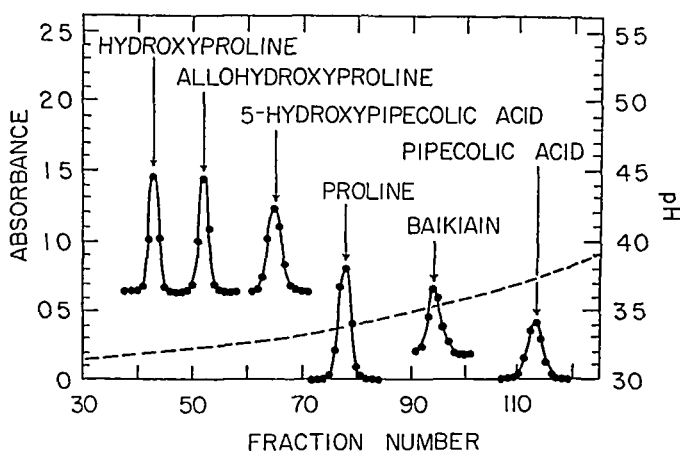


FIG 4 Ion exchange chromatography of a known mixture of cyclic imino acids on a 50 × 0.9 cm column of Dowex 50-X12, minus 400 mesh The 1 ml fractions through 58 were analyzed by Procedure A, the rest by Procedure B The tubes in the region of hydroxyproline (0.801  $\mu$ mole), allohydroxyproline (0.785  $\mu$ mole), and 5 hydroxyproline (0.690  $\mu$ mole) were read at 350 m $\mu$ , proline (0.912  $\mu$ mole) at 510 m $\mu$ , baikian (0.478  $\mu$ mole) at 395 m $\mu$ , and pipecolic acid (0.766  $\mu$ mole) at 565 m $\mu$  A water blank was used The dash line represents the pH gradient

The pH gradient is not necessary, but it is convenient since it speeds the movement of the slower moving imino acids and sharpens the peaks At pH 3.10 on the 50 cm column, pipecolic acid appears in the effluent at about 175 ml compared to about 110 ml when the gradient is used

Not all of the known cyclic imino acids have been examined by these procedures Those not studied include 4-hydroxyproline,  $\gamma$ -methylproline,  $\gamma$ -methyl- $\gamma$ -hydroxyproline, 3,4-dehydropiperidine-3-carboxylic acid, and acetidine-2-carboxylic acid (4) The presence of any of these in a mixture might require a modification of the present ion exchange methods to achieve complete resolution Since each of the imino acids so far examined by Procedure B has given an absorption spectrum unlike any of the others, the presence of a new imino acid would almost certainly be

evident. In fact, the absorption spectrum and the position in the effluent should provide nearly unequivocal proof of identity. Such evidence can be strengthened further by paper chromatography (discussed below).

As has been reported previously for a 100 cm column (14), the diastereoisomers of hydroxyproline separate completely on the 50 cm column. This is not true of the diastereoisomers of 5-hydroxyproline. The presence of both isomers manifests itself by only a slight broadening of the peak. A 150 cm column will separate them enough for analytical purposes (1).

The recoveries of the imino acids from the column are complete within the experimental error. For example, in the experiment shown in Fig. 4, the following per cent recoveries were obtained: hydroxyproline 98, allohydroxyproline 105, 5-hydroxyproline 97, proline 97, bakiam 102, and pipecolic acid 99. The first two recoveries were calculated from calibration curves (Procedure A). The rest were calculated from a standard (in duplicate) analyzed with the samples (Procedure B).

*Interferences*—To examine the effect of the presence of other amino acids, a known mixture of all the amino acids commonly found in protein hydrolysates was chromatographed on the ion exchange column. Three separate aliquots were analyzed, one by Procedure A, the second by Procedure B, and the last by the Moore and Stein ninhydrin method (15), which shows all of the amino acids. Aspartic acid (2  $\mu$ moles) gave a barely visible increase in the blank values following hydroxyproline (Procedure A). Asparagine, which moves with allohydroxyproline, behaved similarly. In large amounts it might be mistaken for allohydroxyproline, except that it does not survive acid hydrolysis. Glutamic acid, which appeared immediately after 5-hydroxyproline, and alanine, which was found in nearly the same position as bakiam, reacted in Procedure B to a very small extent. In each instance the amount of color obtained was considerably less than one-hundredth that given by the cyclic imino acids. None of the other amino acids in this portion of the effluent could be seen in amounts up to 3  $\mu$ moles.

Amino acids that would be found in other portions of the effluent, such as the basic amino acids, and other classes of compounds have not been examined for possible interferences. But it is evident that in general the colorimetric methods cannot be applied directly to complex biological samples without the chromatographic separation. It may be possible in some specific instances.

*Paper Chromatography*—A tracing of a composite two-dimensional paper chromatogram of the cyclic imino acids, together with the common amino acids, appears in Fig. 5. Ninhydrin reacts with the cyclic imino acids on the paper to give distinctive colors, especially when viewed under ultra-

violet light In natural light the hydroxyprolines appear pinkish yellow, fading to brown Prohne and baikiam are yellow, the former fading to brown while the latter is stable for several months in the dark Pipecolic acid and both 5-hydroxypipicolic acids are purple, also fading to brown In ultraviolet light prohne, baikiam, and the hydroxyprolines fluoresce brick-red, the others are a brighter red All of the cyclic imino acids give a blue to green-blue color with isatin The specific colorimetric test devised by Jepson and Smith (16) for hydroxyproline is also given by the allo

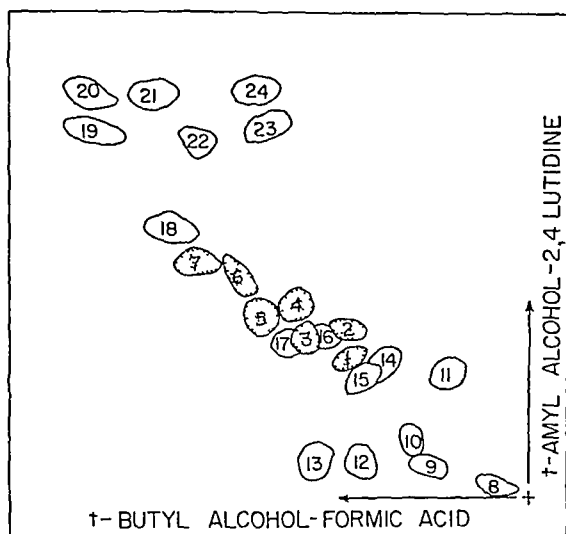


FIG 5 A tracing of a composite, two dimensional paper chromatogram of cyclic imino acids 1, allohydroxyproline, 2, hydroxyproline, 3, allo-5-hydroxypipicolic acid, 4, 5 hydroxypipicolic acid, 5, proline, 6, baikiam, and 7, pipicolic acid The common amino acids shown are 8, cystine, 9, lysine, 10, arginine, 11, histidine, 12, aspartic acid, 13, glutamic acid, 14, serine, 15, glycine, 16, threonine, 17, alanine, 18, valine, 19, isoleucine, 20, leucine, 21, phenylalanine, 22, methionine, 23, tyrosine, and 24, tryptophan

form, but not by the other cyclic imino acids Some of these compounds have been studied by other methods of paper chromatography (4, 5, 17)

These techniques proved valuable as a preliminary guide and to provide confirmation of identity The diastereoisomers of hydroxyproline and 5-hydroxypipicolic acid could be separated on paper In both cases the allo form moved more slowly in the basic solvent <sup>6</sup>

*Cyclic Imino Acids in Dates*—The colorimetric and chromatographic methods were applied to a preliminary study of dates (*cf* Grobbelaar, Pol-

<sup>6</sup> It has recently been shown (B Witkop and C M Foltz, in preparation) that natural 5 hydroxypipicolic acid (from dates), like hydroxyproline, has its functional groups in the trans arrangement

lard, and Steward (17)) Fig 6 shows the analysis of the effluent from a chromatogram of 0.5 gm (wet weight) of the date pericarp (Calavo brand). The total pericarp was hydrolyzed in 3 times its weight of 6N HCl for 24 hours at 105°. The following cyclic imino acids were found in the approximate amounts indicated (mg per gm): hydroxyproline 0.13, 5-hydroxy-pipecolic acid 1.8, proline 0.7, and pipecolic acid 0.005. These substances were identified by their positions in the effluent and their absorption spectra, each was also found on paper chromatograms. A study of dialyzable and non-dialyzable fractions, with and without hydrolysis, showed that hydroxyproline occurred only in combined form in the non-dialyzable portion. Pipecolic acid and 5-hydroxypipecolic acid were found only in the free state. Proline occurred both free and combined.

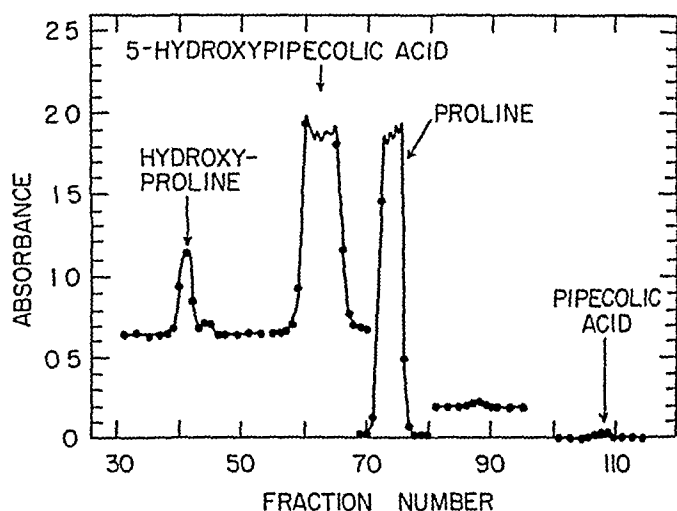


FIG 6 Ion exchange chromatogram of 0.5 gm of acid-hydrolyzed date pericarp. The conditions were the same as those for the known mixture appearing in Fig 4.

The small peak following hydroxyproline is aspartic acid which is present in very high concentrations. The peak following proline results from alanine which also interferes at high concentrations.

The cyclic imino acids in dates have been examined qualitatively by Grobbelaar, Pollard, and Steward (17). The results reported here agree, except that baikiain was not found.

#### SUMMARY

Two colorimetric procedures are described for the quantitative analysis of cyclic imino acids in the effluent fractions from an ion exchange column. Both employ ninhydrin in glacial acetic acid. When the reaction is carried out at room temperature, the method is suitable for hydroxyproline, allohydroxyproline, and proline. Heating at 100° permits the determination of 5-hydroxypipecolic acid, proline, baikiain, and pipecolic acid.

The separation of these compounds by ion exchange and paper chromatography is described. The diastereoisomers of hydroxyproline and 5-hydroxy-pipecolic acid can be separated by paper chromatography and, in the former case, by the ion exchange method.

These methods were employed to study the cyclic imino acids of dates. Hydroxyproline, proline, 5-hydroxy-pipecolic acid, and pipecolic acid were found and their approximate amounts were estimated.

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# OCCURRENCE OF THE 8-METHYL ETHER OF XANTHURENIC ACID IN NORMAL HUMAN URINE\*

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During the development of a chromatographic procedure for the determination of urinary kynurenic acid (1), it was found that both kynurenic and xanthurenic acids could be removed from Dowex 50 (H<sup>+</sup>) columns by washing with a large volume of water<sup>1</sup>. When human urine was passed through Dowex 50 columns, paper chromatography of the concentrated water effluents revealed an additional fluorescent spot. This additional compound has been identified as the 8-methyl ether of xanthurenic acid. This methyl ether has been found in every human urine studied and its excretion invariably increased after the ingestion of L-tryptophan.

## Methods

*Reagents*—Xanthurenic acid 8-methyl ether was prepared by alkaline hydrolysis of ethyl 8-methoxy-4-hydroxyquinaldate (2), m p<sup>2</sup> 240–241°

C <sub>11</sub> H <sub>9</sub> O <sub>4</sub> N	Calculated	C 60.27, H 4.14
	Found <sup>3</sup>	" 60.26, 60.37, H 4.27, 4.18

The corresponding 8-ethyl ether was prepared by a similar method, m p 240–241°

C <sub>12</sub> H <sub>11</sub> O <sub>4</sub> N	Calculated	C 61.80, H 4.76
	Found	" 61.74, 61.82, H 4.73, 4.73

8-Methoxy-4-chloroquinaldic acid was prepared by treating 8-methoxy-4-hydroxyquinaldic acid with POCl<sub>3</sub> (3), m p 130–133°

\* Supported in part by the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, the Wisconsin Division of the American Cancer Society, the American Cancer Society Institutional Grant No. 71A, and the National League Baseball Club of Milwaukee, Inc.

† Scholar in Cancer Research of the American Cancer Society

<sup>1</sup> Price, J. M., unpublished data

<sup>2</sup> All of the melting points were uncorrected. These compounds all decomposed at the melting point. They were all placed in the block about 20° below the melting point and heated at 2° per minute. 8-Methoxy-4-chloroquinaldic acid resolidified at about 138°.

<sup>3</sup> All elemental analyses were made by the Clark Microanalytic Laboratory, Urbana, Illinois



$C_{11}H_9O_3NCl$  Calculated, Cl 14.92, found, Cl 15.24, 15.12

8-Ethoxy-4-chloroquinolinaldic acid (m p 159–160°) was prepared in the same manner

$C_{12}H_{10}O_3NCl$  Calculated, Cl 14.09, found, Cl 14.02, 13.81

Xanthurenic acid was prepared by heating 0.5 gm of its methyl ether in a mixture of 50 ml of glacial acetic acid and 10 ml of 57 per cent HCl at reflux temperature for 5 days

6-Hydroxykynurenic acid was prepared according to Makino and Takahashi (4). Its 6-methyl ether (m p 278°) was prepared by alkaline hydrolysis of ethyl 6-methoxy-4-hydroxyquinolaldate (4). Kynurenic acid was available from material used in previous studies (1).

All of the synthetic compounds were purified until they reached constant melting points and showed single fluorescent spots on paper chromatography in the five solvent systems used (Table I).

*Isolation of Quinoline Compounds from Urine*—5 per cent of a 24 hr human urine was diluted to 120 ml with  $H_2O$  and 30 ml of 1 N HCl was added. The acidified urine was passed through a  $0.9 \times 3.0$  cm column of Dowex 50 ( $H^+$ ) (1) and the column was washed with 50 ml of 0.2 N HCl, 100 ml of 0.5 N HCl, and 60 ml of  $H_2O$ . The quinoline compounds were then eluted with 400 ml of  $H_2O$ . The water was removed *in vacuo* with the aid of a water bath at 55° and the residue was dissolved in 1 ml of 0.1 N  $NH_4OH$  for paper chromatography.

For the isolation of larger quantities of these compounds from urine, the above procedure has been used on 200 times this scale to handle ten 24 hour urine samples. To simplify the large scale procedure, the quinoline compounds were collected from the water effluent from the Dowex 50 by passing the solution through a  $5.6 \times 6.1$  cm column of Dowex 1 formate. The quinoline derivatives were eluted from the formate column with 2 liters of 10 N  $HCOOH$ . The acid eluate was evaporated to dryness *in vacuo* on a water bath at 55°.

*Separation of Kynurenic Acid, Xanthurenic Acid, and Xanthurenic Acid Methyl Ether*—The three synthetic quinoline compounds were eluted from  $1.3 \times 16$  cm columns of Dowex 1 formate (5) by a gradient elution (6) with formic acid. With 250 ml of  $H_2O$  in the mixer and 600 ml of 10 N  $HCOOH$  in the reservoir, 200 fractions of 3.0 ml each were collected in 20 hours. A single drop from each tube from Tubes 80 to 180 was spotted on filter paper, dried, exposed to  $NH_3$  fumes, and examined at once with ultraviolet light. Kynurenic acid was revealed as a dark, absorbing spot while xanthurenic acid and its 8-methyl ether showed bright yellow and brilliant light blue fluorescence, respectively. Tubes 88 to 105 contained

kynurenic acid, while xanthurenic acid ether was found in Tubes 114 to 132, and xanthurenic acid was present in Tubes 142 to 160. The columns would handle no more than 5 mg of any one of the three compounds.

The mixture of quinoline compounds from urine was dissolved in 0.1 N  $\text{NH}_4\text{OH}$  for application to the Dowex 1 formate columns. After washing the column with 200 ml of  $\text{H}_2\text{O}$ , the urinary quinoline compounds were eluted as described. The appropriate fractions were pooled and evaporated to dryness *in vacuo* on a water bath at  $55^\circ$ .

The xanthurenic acid derivative isolated from ten 24 hour urine samples was divided into three portions. The dried residue from one portion was heated for 6 hours under reflux with 1 ml of 57 per cent HI. Another portion was heated with 1 ml of  $\text{POCl}_3$  for 2 hours to make the 4-chloro derivative. The reagents were removed *in vacuo* and the contents of the flasks were dissolved in 0.5 ml of 0.1 N  $\text{NH}_4\text{OH}$  for paper chromatography. The remaining portion of the compound isolated from urine was used for the ultraviolet spectra and paper chromatography.

*Paper Chromatography*—The details of the paper chromatography are given in footnotes to Table I. The diazotized sulfanilic acid was used for spraying the chromatograms as described by Dalghesh (8). All chromatograms were also sprayed with 1.7 per cent  $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ , followed by 5 per cent  $\text{NaHCO}_3$ .

### Results

It was apparent (Table I) that the new urinary quinoline compound was indistinguishable from synthetic xanthurenic acid 8-methyl ether.

The ultraviolet spectrum of the new urinary product was almost identical with that of the synthetic 8-methyl ether of xanthurenic acid in 0.1 M phosphate buffers at pH 2.0, 7.4, and 12.0.

When aliquots of 24 hour urine collections from normal human subjects were applied to Dowex 50 columns and the columns were washed with water as described, paper chromatograms of the concentrated water effluents always revealed spots corresponding to kynurenic acid, xanthurenic acid, and the 8-methyl ether of xanthurenic acid. Ingestion of 2.0 gm of L-tryptophan was always followed by an increase in the intensity of the fluorescence of the spots corresponding to each of these three compounds. It was estimated that the tryptophan supplements increased the excretion of the methyl ether to 2 to 5 times the basal levels.

When the procedure was applied to normal dog urine, it was possible to detect kynurenic and xanthurenic acids. Cat urine contained a trace of kynurenic acid, but the methyl ether of xanthurenic acid could not be detected in either cat or dog urine.

When the water effluents from the Dowex 50 columns were chromato-

## XANTHURENIC ACID ETHER

TABLE I  
Results of Paper Chromatography of Synthetic and Natural Quinoline Compounds and Some of Their Derivatives\*

Synthetic (S), natural (N), or derivative of natural (DN) quinoline compound†															
Reaction with DSA† Reaction with FeNH <sub>4</sub> (SO <sub>4</sub> ) <sub>2</sub> Acetic Mason-Berg system, ‡ R <sub>F</sub> Neutral Mason-Berg system, ‡ R <sub>F</sub> NH <sub>4</sub> OH Mason-Berg system, ‡ R <sub>F</sub> Dalghesh system, § R <sub>F</sub> Butanol + 0.2 N NH <sub>4</sub> OH, § R <sub>F</sub> Fluorescence	KA (S)	KA (N)		XA (S)		XA (N)		MXA (S)		MXA (N)		EXA (S)	CIXA (S)	CINXA (DN)	CIEXA (S)
				Red Green	0 58 0 64 0 60 0 55 0 19 Yellowish green	Red Green	0 58 0 64 0 63 0 40 0 54 0 03 Grayish lavender	Red Green	0 58 0 63 0 68 0 66 0 56 0 25 Light blue	Yellow, fades 0 63 0 68 0 66 0 56 0 25 Light blue	Yellow, fades 0 63 0 68 0 66 0 56 0 33 Light blue				
	0 60	0 60		0 58		0 58		0 68		0 68		0 72		0 71	0 85
	0 63	0 64		0 64		0 63		0 66		0 66		0 70		0 71	0 80
	0 60	0 60		0 40		0 63		0 56		0 56		0 69		0 70	0 77
	0 55	0 55		0 54		0 53		0 25		0 25		0 83		0 82	0 87
	0 19	0 17		0 03		0 02		0 33		0 33		0 38		0 39	0 48
	Yellowish green	Yellowish green	Grayish lavender	Grayish lavender	Grayish lavender	Grayish lavender	Light blue	Light blue	Light blue	Light blue	Light blue	Light blue	Light blue	Light blue	Light blue

\* Whatman No 1 filter paper was used for all paper chromatograms

† The following abbreviations are used KA, kynurenic acid, XA, vanthurenic acid, MXA, vanthurenic acid, CIXA, 8-methoxy-4-chloroquinoline acid, CIEXA, 8-methoxy-4-chloroquinoline acid

‡ The solvent system of Mason and Berg (7) consisted of methanol and ammonium modifications 1 ml of glacial acetic acid and 1 ml of water

§ Dalghesh (9) used the solvent system of methanol and ammonium modifications 1 ml of glacial acetic acid and 1 ml of water

\* Whatman No. 1 filter paper was used for all paper chromatograms

† The following abbreviations are used: KA, kynurenic acid; XA, xanthurenic acid; MXA, xanthurenic acid 8-methyl ether; CINXA, 8-ethoxy-4-chloroquinoline acid; CUEXA, 8-ethoxy-4-chloroquinoline acid, DSA,

and ammonium modifications

‡ The solvent system of Mason and Berg (7) consisted of methanol, butanol, benzene, and water (2:1:1:1). For the acetic acid

§ Dalghesh (8) used the organic phase of butanol, acetic acid, and water (1:1:5) for a solvent system. This solvent and the

graphed on Dowex 1 formate as described, the three urinary quinoline compounds were eluted with formic acid as were the corresponding synthetic metabolites. Concentrates of the three fractions gave single spots on paper chromatography.

The uncleaved methyl ether of xanthurenic acid was readily detected by paper chromatography in impure samples of synthetic xanthurenic acid. Samples of xanthurenic acid prepared in several laboratories were found to contain this impurity which has been considered to be xanthurenic acid (7) in paper chromatographic studies. Because of the limited solubilities of these two quinoline compounds in formic acid, it was not convenient to utilize chromatography on Dowex 1 formate for the purification of large amounts of synthetic xanthurenic acid. However, the methyl ether could be completely cleaved to xanthurenic acid by refluxing HI and acetic acid in 5 to 8 days. Since the length of time required to cleave the last traces of the methyl ether varied, the progress of the reaction was always followed by paper chromatography.

No conclusive evidence was obtained for the presence of 6-hydroxykynurenic or 6-methoxykynurenic acids on paper chromatograms of the water effluents obtained after passing human urine through Dowex 50 columns. Although the fluorescence of 6-methoxykynurenic acid was similar to that of xanthurenic acid methyl ether, it clearly separated from the latter in several of the paper chromatographic systems.

#### DISCUSSION

Lederer (9) recently called attention to the fact that methylation of phenolic hydroxyl groups had not been observed in animals, although methyl ethers have been found in numerous plant products. Shortly thereafter MacLagan and Wilkinson (10) found that butyl-4-hydroxy-3,5-dinitrobenzoate was partly metabolized by man to the corresponding methyl ether. Rats and rabbits failed to excrete the methoxy derivative.

Lederer and Polonsky (11) isolated ferulic acid and some chemically similar methyl ethers from horse urine. More recently Armstrong, Shaw, and Wall (12) have detected several of the same methyl ethers in human urine. Although it is possible that these phenolic ethers were derived from methyl ethers in dietary plant material (9, 12), the observations of MacLagan and Wilkinson (10) and the present studies make it increasingly likely that such compounds may be metabolic products in animals.

Studies are now in progress concerning the mechanism and site of synthesis of xanthurenic acid methyl ether in animals.

#### SUMMARY

A compound identified as the 8-methyl ether of xanthurenic acid was found in normal human urine. It was present in quantities of perhaps

less than 1 mg in every 24 hour human urine studied and the excretion of this methyl ether invariably increased after the ingestion of L-tryptophan. Xanthurenic acid 8-methyl ether appears to be the first example of the excretion of a methyl ether by an animal following the ingestion of a normal nutrient.

A procedure was described for the purification and separation of xanthurenic acid, xanthurenic acid, and xanthurenic acid methyl ether from urine.

The syntheses of 8-methoxy- and 8-ethoxy-4-hydroxyquinaldic acids, as well as 8-methoxy- and 8-ethoxy-4-chloroquinaldic acids, were reported.

The authors wish to thank Mr. Meredith L. Nelson for valuable assistance with the synthetic work.

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# THE CONVERSION OF KYNURENIC ACID TO QUINALDIC ACID BY HUMANS AND RATS\*

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The ingestion of large doses of L-tryptophan by humans and dogs was followed by the urinary excretion of increased amounts of a compound chromatographically and spectroscopically identical with quinaldic acid (1). Subsequently it was observed that ingestion of kynurenic acid by man led to the excretion of comparatively large amounts of this same metabolite, which has now been isolated in crystalline form and identified as quinaldic acid.

## Methods

Kynurenic acid used in previous studies (1) was suspended in water for ingestion by human subjects and administration to rats by stomach tube. Urine collections were made under toluene in amber bottles.

Quinaldic acid was isolated from human urine as previously described (1), but on a scale 40 times as large in order to handle 48 hour urine collections. After removal of the 3.0 N HCl *in vacuo* on a water bath at 55–60°, the residue was dissolved in 0.2 N HCl, adsorbed on a 1.3 × 20.5 cm column of Dowex 50 (H<sup>+</sup>), and removed by gradient elution (2). The reservoir contained 600 ml of 6 N HCl and the mixer 250 ml of H<sub>2</sub>O, and the 3.0 ml fractions in Tubes 100 to 185 were examined by ultraviolet spectrophotometry for the presence of quinaldic acid. The appropriate fractions were pooled and evaporated as before, and the residue was dissolved in 0.2 N NH<sub>4</sub>OH for application to a 1.3 × 16 cm column of Dowex 1 formate (3). After another gradient elution (reservoir, 600 ml of 4 N HCOOH, mixer, 250 ml of H<sub>2</sub>O, 3.0 ml fractions), Fractions 46 to 80 were pooled and evaporated to dryness. Colorless crystals were obtained from the residue after recrystallization from benzene. The quinaldic acid from rat urine was purified chromatographically, but crystals were not obtained.

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† Scholar in Cancer Research of the American Cancer Society.

The quinaldic acid was decarboxylated (4) and the quinoline was isolated as the picrate. The ultraviolet spectra of natural and synthetic quinaldic acids were compared with the aid of a Beckman model DK spectrophotometer. The methods for paper chromatography (5) and for the quantitative determinations of the various tryptophan metabolites were those previously described (1, 6, 7).

### Results

The urinary excretion of kynurenic and quinaldic acids before and after ingestion of a single dose of kynurenic acid is shown in Table I. Ingestion

TABLE I  
*Average Daily Urinary Excretion in Micromoles of Kynurenic Acid (KA) and Quinaldic Acid (QA) after Ingestion of Single Dose of Kynurenic Acid*

No. of subjects	Dose KA	Metabolite	1st day before ingestion	Days after KA ingestion		
				1	2	3
3*	820	KA	14	118	28	17
		QA	5	20	39	7
2*	410	KA	11	61	21	18
		QA	4	31	67	33
1*	164	KA	14	30	12	14
		QA	6	30	29	5
2†	164	KA	0.3	7.0	1.4	0.5
		QA	0.3	4.5	2.0	1.0

\* Humans

† Rats

of kynurenic acid failed to alter the urinary excretion of xanthurenic acid, *N*-methyl-4-quinolone, 4-quinolone, *N*-methyl-2-pyridone-5-carboxamide, anthranilic acid glucuronide, anthranilic acid, *o*-aminohippuric acid, acetyl kynurenine, or kynurenine by humans or rats.

After ingestion of 820  $\mu$ moles (155 mg) of kynurenic acid, 37 and 25 mg of colorless crystals of quinaldic acid were isolated from 48 hour urine collections from two subjects. One subject ingested 410  $\mu$ moles of kynurenic acid on another occasion and 13 mg of quinaldic acid were obtained. The crystals melted<sup>1</sup> at 155° (4) with decomposition, either alone or when mixed with authentic quinaldic acid. The picrates of the decarboxylated natural or authentic quinaldic acids melted at 200° alone or mixed with authentic quinoline picrate.

<sup>1</sup> All melting points were uncorrected.

The spectra of natural quinaldic acid suggested that the isolated material was the known dihydrate (4), and drying over  $P_2O_5$  at  $100^\circ$  resulted in loss of weight equivalent to 2 moles of water. The spectra of each preparation of the natural product in 0.1 M phosphate buffers at pH 2.0, 7.4, and 12.0, and in 3.0 N HCl were identical with the spectra of synthetic quinaldic acid.

On paper chromatograms quinaldic acid was revealed as a dark purple spot under ultraviolet light. The authentic and natural compounds did not separate when chromatographed together and had identical  $R_F$  values of 0.83 and 0.71 in the acidic and alkaline solvent systems (5), respectively. Quinaldic acid was also detected in the purified fraction from rat urine with the aid of paper chromatography.

#### DISCUSSION

Since the ingestion of L-tryptophan was followed by the urinary excretion of a substance which was chromatographically and spectrophotometrically identical with quinaldic acid (1), it would appear that kynurenic acid formed from tryptophan was converted to quinaldic acid in a manner similar to administered kynurenic acid.

Ellinger and Matsuoka (8) found that quinaldic acid was not oxidized to kynurenic acid by rabbits. They did not mention attempts to demonstrate the reverse reaction.

Preliminary studies have shown that orally ingested kynurenic acid-carboxyl- $C^{14}$  was excreted in the urine of man and rats in part as quinaldic acid-carboxyl- $C^{14}$ . After the addition of kynurenic acid-carboxyl- $C^{14}$  and non-radioactive quinaldic acid to normal human urine, the quinaldic acid which was isolated was not radioactive.<sup>2</sup>

Quinaldic acid appears to be a new natural product, and its formation by the dehydroxylation of kynurenic acid apparently represents a new biochemical reaction.

#### SUMMARY

As much as 29 per cent of a single oral dose of kynurenic acid was accounted for in the urine of human subjects as quinaldic acid. The same metabolite was detected in rat urine after ingestion of kynurenic acid.

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# GASTROINTESTINAL DIGESTION OF STARCH

## II PROPERTIES OF THE INTESTINAL CARBOHYDRASES\*

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In terminal starch digestion in the small intestine, the enzymes oligo-1,6-glucosidase and maltase ( $\alpha$ -glucosidase) have been shown to complete the hydrolysis of the saccharides left after  $\alpha$ -amylase action (1). In a previous paper, oligo-1,6-glucosidase has been identified, partially separated from maltase, and differentiated from the polysaccharide debranching enzyme amylo-1,6-glucosidase (1). In this paper some of the properties of the intestinal carbohydrases are presented. Particular attention has been paid to the possible importance of histidine in enzymatic hydrolysis. Some of this work has been the subject of a preliminary report (2). The properties of amylo-1,6-glucosidase are reported in another communication (3).

### *Materials and Methods*

*Enzymes*—Oligo-1,6-glucosidase was prepared essentially as previously described (1). It has been found that higher yields are obtained when the enzyme is fractionated in the presence of Versene. Accordingly, 0.001 M Versene was included in all aqueous solvents used.

Solid ammonium sulfate was used during the fractionation in place of a saturated solution. Solid  $\text{KHCO}_3$  was used to adjust the pH to 7 after ammonium sulfate addition. Unless otherwise stated, fractions in these experiments were 18 to 44 per cent ethanol fractions. The preparation of maltase essentially free of oligo-1,6-glucosidase activity is described in a later section. Other enzymes were prepared by methods already cited (1).

### *Coenzymes and Substrates and Inhibitors*

The sources or methods of preparation of coenzymes and substrates have been listed (1). Tris(hydroxymethyl)aminomethane (Tris), purchased from the Sigma Chemical Company, was sublimed before use. *tert*-Butylamine, 2-amino-2-methyl-1-propanol, and 2-amino-2-methyl-1,3-propanediol were purchased from the Eastman Kodak Company. The first two

\* Supported in part by a grant from the National Science Foundation.

were redistilled before use, and the last was recrystallized twice from aqueous acetone. Glycylglycine was purchased from the Sigma Chemical Company. When it was found that some batches were inhibitory in the spectrophotometric assay, the dipeptide was synthesized by the method of Schott and collaborators (4) and recrystallized from aqueous ethanol. Tetramethyl pyrophosphate (TMP) was kindly supplied by C. W. Kern, Department of Entomology, and diisopropyl fluorophosphate (DIP) by B. J. Jandorf and W. H. Summerson, Army Chemical Center, Maryland.

### *Analytical*

Activities of intestinal carbohydrases were determined spectrophotometrically, as previously described (1), with the following modifications. Reaction mixtures were decreased from 3 to 0.9 ml. and run in 1 ml. cylindrical cells with 1 cm. light path. By reducing the amount of glycylglycine buffer and by increasing the ratio of glucose-6-phosphate dehydrogenate to hexokinase, a  $3\frac{1}{2}$ -fold increase in activity has been obtained. A typical reaction mixture is given in Fig. 2. Except when otherwise stated, all  $\Delta$  will refer to a change in optical density of 0.001 per minute as previously defined (1) with the 0.9 ml. reaction mixture.

Protein was routinely determined by trichloroacetic acid precipitation (1). In some cases (Table I) the biuret method of Robinson and Hogden (5), the phenol method of Lowry and collaborators (6), and the ultra violet absorption at 280 m $\mu$  (7) were also used. Bovine plasma albumin served as standard with all of the methods employed.

For deproteinization the  $\text{Ba}(\text{OH})_2\text{ZnSO}_4$  method of Somogyi (8) was used. Reducing power was determined by methods previously detailed (1).

### *Particulate Nature of Enzymes*

The particulate nature of the intestinal carbohydrases was suspected when it was found that enzymatic activity decreased upon prolonged high speed centrifugation. In addition, sharp fractionation with ammonium sulfate has not been achieved and attempts to elute enzyme adsorbed on calcium phosphate and alumina gels were not satisfactory quantitatively. The effect of repeated freezing and thawing of intestine on the specific activity of mucosal extracts was therefore studied. Sections from the same intestinal segment were used for preparation of extracts. All the steps were carried out at 3° under similar conditions. 10 gm. portions of mucosa (Table I) were extracted with equal weights of sand and 5 volumes of 0.9 per cent NaCl-0.001 M Versene, pH 7.0 (hereafter referred to as saline-Versene). Extracts were centrifuged in the International refrigerated centrifuge (model PR-2) with the multispeed head at highest speed for 30 minutes, and filtered through Whatman No. 1 paper. Dialysis was

carried out for 5 hours against saline-Versene to lower the endogenous glucose content prior to assay. Specific activities (Table I) of maltase and oligo-1,6-glucosidase increased more than 3-fold after freezing and thawing the intestine twice.

To identify the particulate nature of these carbohydrases further, differential centrifugation experiments were performed by a procedure modified from Morton (9) (Table II). Intestines collected at the slaughterhouse were received in the laboratory, packed in ice within several hours after slaughter. The contents were first rinsed with 0.25 M sorbitol-0.001 M Versene, pH 7.4 (hereafter referred to as sorbitol-Versene), sections were slit open, and mucosa were removed by scraping with a knife. A 20 gm

TABLE I  
*Effect of Freezing and Thawing on Activity\* of Intestinal Carbohydrases*

Preparation	Volume	Oligo- 1,6-glucosidase	Maltase	Protein†	Specific activity	
					Oligo- 1,6-glucosidase	Maltase
	ml	units per ml	units per ml	mg per ml	units per mg	units per mg
Fresh	44	72	436	8.8	8.2	49.5
Frozen and thawed (once)	44	104	944	7.9	13.2	119.5
" " " (twice)	44	220	1652	8.8	25.0	188.0

\* Activity determined as previously described (1).

† By trichloroacetic acid precipitation method. In a previous experiment, protein concentration was determined by trichloroacetic acid precipitation, biuret color, ultraviolet absorption, and phenol methods with good agreement. After freezing and thawing two times, protein analysis by ultraviolet absorption was about 15 per cent higher than by the other methods.

portion of mucosa was suspended at 0° in 100 ml of sorbitol-Versene and gently stirred manually for 15 minutes. The initial extract was prepared by centrifugation in the Servall model SS-1 at  $2500 \times g$  for 20 minutes. Fractions B and C were prepared in the Servall model SS-1 and Fractions D and E in the Spinco model L centrifuges. In order to lower endogenous glucose, all fractions were dialyzed against saline-Versene for 15 hours prior to assay. From 18.9 to 29.4 per cent of the maltase and oligo-1,6-glucosidase activities sediment under conditions required to sediment mitochondria ( $14,000 \times g$  for 15 minutes) (10). From 81.3 to 91.3 per cent of the enzymatic activities remained in the supernatant fluid (Fraction C).<sup>1</sup> With stronger centrifugal force, in the case of maltase, activity was dis-

<sup>1</sup> The recovery of oligo-1,6-glucosidase activity greater than 100 per cent in Fractions B and C is consistent with an activation of the enzyme during the preparation, possibly by a release of the enzyme from its particulate form.

tributed between small particles and supernatant fluid. In the case of oligo-1,6-glucosidase, no activity was present in the supernatant fluid whereas remaining activity was associated with small particles. Recuspension of these particles in sorbitol-Veisene and recentrifugation at  $79,000 \times g$  for 60 minutes gave a 31.9 per cent recovery of original oligo-1,6-glucosidase activity associated with the particles with no activity in the supernatant liquid. With maltase, 33 per cent of original activity was recovered in the particles and 9.3 per cent in the supernatant liquid which indicated that activities were not removed from particles by washing. The loss of the more labile oligo-1,6-glucosidase activity with high speed centrifugation has been noted in all experiments run and is as yet unexplained. Recombination of Fractions E and B resulted in only addi-

TABLE II  
*Differential Centrifugation of Intestinal Extracts*

Fraction	Method of preparation	Volume	Activity		Recovery	
			Oligo-1,6-glucosidase	Maltase	Oligo-1,6-glucosidase	Maltase
		ml	units per ml	units per ml	per cent	per cent
A	Initial extract	83	430	4060		
B	$14,000 \times g$ 15 min ppt	18	584	3560	29.4	18.9
C	$14,000 \times$ " 15 " supernatant fluid	83	394	3300	91.3	81.3
D	$79,000 \times$ " 60 " ppt	16	580	5000	47.3	43.2*
E	$79,000 \times$ " 60 " supernatant fluid	46	0	1070	0	26.5*

\* An aliquot (46 ml) of Fraction C was taken for centrifugation. The per cent of recovery was calculated on the basis of the original volume of Fraction C.

tive activity. It is of interest that a bacteriophage of *Azotobacter* has been reported to be inactivated by the forces of high speed centrifugation (11). From this experiment it is evident that the major fraction of both enzymatic activities is associated with the small particles which sediment as microsomes. As mentioned previously (1), intestinal extracts which are kept at  $5^\circ$  for extended periods occasionally show 2- to 3-fold increases in the specific activity of oligo-1,6-glucosidase with no decrease in protein concentration. This is presumably due to a release of enzymes from the small particles.

#### *Preparation of Maltase Free from Oligo-1,6-glucosidase*

Upon fractionation of acetone powder extracts of hog intestinal mucosa preparations of maltase essentially free from oligo-1,6-glucosidase activity have been made. A 150 gm portion of mucosa (Table III) was homogenized at  $-12^\circ$  in a Waring blender with 225 ml of acetone (pre-chilled to

-12°) The homogenate was poured into a beaker containing 1 liter of acetone, stirred, and rapidly filtered by suction at 3° The powder was further dried by rolling between large sheets of blotter paper It was then placed in a vacuum desiccator at room temperature over a mixture of P<sub>2</sub>O<sub>5</sub> and paraffin oil until the odor of acetone was no longer detectable (several hours) The light tan powder was stored under a vacuum at 3°

All subsequent steps were carried out in the cold room at 3° A 5 gm portion of acetone powder was extracted with 50 ml of saline-Versene The clear reddish extract obtained after centrifugation was fractionated with solid ammonium sulfate, and the fraction precipitating between 0.35 and 0.80 saturation was collected During the fractionation the pH was adjusted to 7 by the addition of solid KHCO<sub>3</sub> The ammonium sulfate

TABLE III  
*Separation of Maltase from Oligo-1,6-glucosidase*

Fraction	Volume	Activity*		Protein	Specific activity		Ratio, maltase-glucosidase
		Maltase	Oligo-1,6-glucosidase		Maltase	Oligo-1,6-glucosidase	
	ml	units per ml	units per ml	mg per ml	units per mg	units per mg	
Initial	38.0	840	122	11.7	71.8	10.4	6.9
0.35-0.80 saturated ammonium sulfate	28.6	540	30	6.8	79.5	4.4	18.0
Alumina gel supernatant fluid	27.0	416	34	6.2	67.1	5.5	12.2
32-46% ethanol	10.0	440		0.99	445.0		
46-59% "	10.5	1032		30.0	345.0		

\* The activity was determined as previously described (1)

fraction was dissolved in a small volume of saline-Versene and diluted to 1 per cent protein concentration. Alumina C<sub>γ</sub> was added slowly with stirring to a gel-protein ratio of 0.15. The supernatant liquid obtained after centrifugation was fractionated with 95 per cent ethanol at -2° to -5°. Fractions precipitating between 32 and 46 per cent and 46 to 59 per cent were collected, dissolved in small volumes of saline-Versene, and dialyzed for 2 to 3 hours against the same solution to remove ethanol. Although only a 6-fold purification of maltase was achieved (Table III) fractions were essentially free of oligo-1,6-glucosidase activity<sup>2</sup>. As yet no preparation of the more labile oligo-1,6-glucosidase free of maltase has been prepared. Seiji (12) was unable to separate these activities by fractionation of acetone powders.

<sup>2</sup> One of the three preparations made in this way had traces of oligo-1,6-glucosidase activity.

was not eliminated by running the determinations at constant temperature (Fig. 3)

Following Dixon (15), plots of  $-\log K_m$  ( $pK_m$ ) against pH for both enzymes have been made (Figs. 2 and 3). At acid pH,  $pK_m$  is essentially independent of pH, while, at alkaline pH,  $pK_m$  decreases with increase in pH. In the case of maltase at alkaline pH, the plot has a slope calculated as  $-0.91$ , while with oligo-1,6-glucosidase the slope has been calculated as  $-1.4$ .<sup>5</sup> In both cases the change in slope is approximately between pH

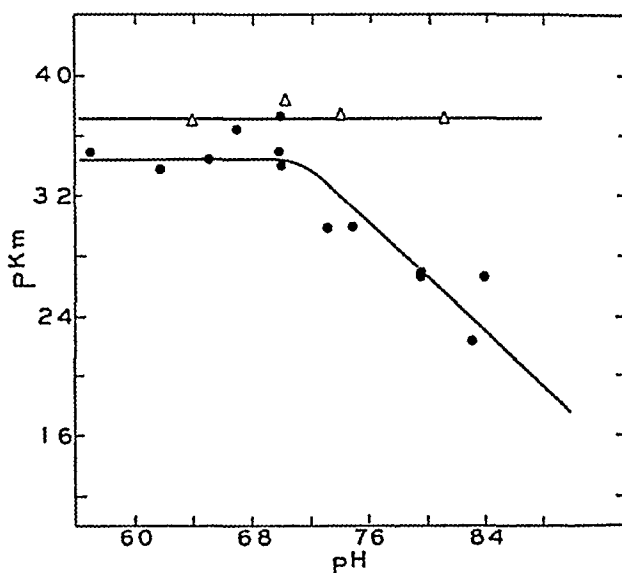


FIG. 2 pH dependence of  $pK_m$  for maltase.  $\Delta$ , control runs with glucose and hexokinase, glucose-6-phosphate dehydrogenase system. Reaction mixtures as previously described (1).  $\bullet$ , maltase reaction mixtures contained glycylglycine buffer, 0.25 M, 0.2 ml,  $Mg^{++}$ , 0.3 M, 0.03 ml, glucose-6-phosphate dehydrogenase, 0.1 mg of lyophilized powder per ml, 0.1 ml, hexokinase, 2.8 per cent (20 per cent pure), 0.01 ml, TPN, 0.005 M, 0.03 ml, ATP, 0.01 M, 0.03 ml, maltase, 25 units (oligo-1,6-glucosidase-free), volume, 0.9 ml. Reactions run at room temperature.

6.9 and 7.2. A  $pK_m$ -pH plot for amylo-1,6-glucosidase has been reported (3). With this latter enzyme a curve of similar slope was obtained, with a change of slope at about pH 7.5 to 7.6.

Control experiments with the hexokinase, glucose-6-phosphate dehydrogenase system, and glucose as substrate are shown in Fig. 2. Over the pH range studied (6.4 to 8.05), the apparent  $pK_m$  was independent of pH.

<sup>5</sup> Lines were fitted to points by the method of least squares. In the case of maltase, nine points, including and more alkaline to pH 6.98, were used, in the case of oligo-1,6-glucosidase, twelve points, including and more alkaline to pH 6.95, were used. Both lines were proved to be statistically significant by the method of Koller (16).

Since the substrate is uncharged and glycylglycine has two  $pK_a$  values of 3.2 and 8.07 (17), the changes in slope observed indicate that ionizable enzymatic groups with  $pK_a$  values of approximately 7 are implicated in combination of enzyme and substrate. This is in agreement with the analysis of Dixon (15), which indicates that a change in slope in the  $pK_m$ -pH plot in the negative direction denotes ionization in either free enzyme or free substrate.

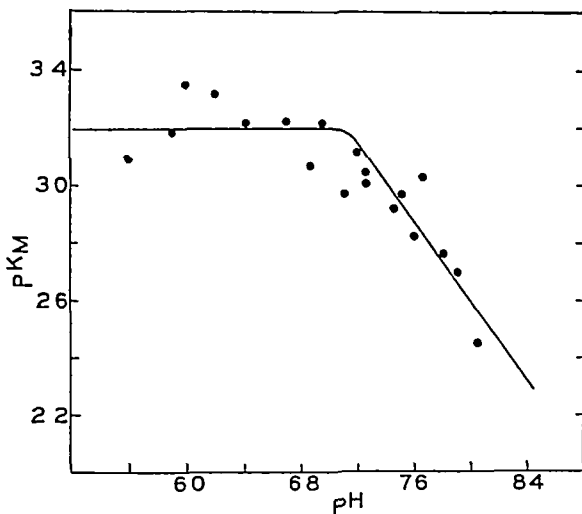


FIG 3 pH dependence of  $pK_m$  for oligo-1,6-glucosidase. Reaction mixtures were as shown in Fig 2. Reactions run at 30° in a Beckman model DU spectrophotometer equipped with a cell carriage through which water at constant temperature was circulated.

### *Methylene Blue Photooxidation*

Weil and Buchert have shown that photooxidation of proteins in the presence of methylene blue leads to a destruction of photosensitive amino acids, histidine being the most sensitive (18). Photooxidations of intestinal extracts were carried out in the Warburg apparatus at 20° under conditions similar to those described by Weil and Buchert. In each experiment, control vessels containing water instead of methylene blue in the side arms were run. The first three experiments (Table V) were performed in an apparatus illuminated from below by a bank of fluorescent lights<sup>6</sup>. The last five were performed in a circular Warburg apparatus lighted from above by a ring of six incandescent bulbs, each 150 watts, and cooled from the side by an electric fan. Determinations of activity were performed at the end of each run by spectrophotometric assay. In each case methylene

<sup>6</sup> We are indebted to Dr R. Emerson of the Botany Department for the use of this apparatus.



blue was added to aliquots of control reaction mixtures in amounts equal to that present in experimental reaction mixtures prior to determination of activity. Addition of methylene blue to controls had little or no effect on enzymatic activity.

For amino acid determinations, protein samples were placed in sealed tubes in 3 N HCl and hydrolyzed in the autoclave for 5 hours at 15 pounds pressure. Before HCl was added, protein samples were lyophilized to dryness. After hydrolysis the tubes were opened and the contents neutralized and made up to volume, then analyzed for histidine, tyrosine, leucine, and lysine by microbiological assay with *Leuconostoc mesenteroides*.

TABLE V  
*Photooxidation of Intestinal Extracts with Methylene Blue*

pH	Gas phase	Extract added	Time	Net oxygen uptake	Activity remaining		Amino acid remaining		
					Oligo-1,6-glucosidase	Maltase	Histidine	Tyrosine	Tryptophan
		mg protein	min	$\mu$ l	per cent	per cent	per cent	per cent	per cent
7.4	O <sub>2</sub>	16.2	40	43	85.2				
8.4	"	10.9	95	87	39.7	76.2			
8.6	"	15.6	135	154	32.0	58.7	19.6	74.1*	
8.6	"	13.8	120	82	35.6	59.0			
8.6	"	14.2	60	56	66.7	71.6	39.8	84.5	83.0
8.6	"	14.2	130	96	49.0	61.6	25.8	76.1	72.0
8.6	"	14.2	180	104	42.2	57.6	19.6	65.5	63.0
8.6	N <sub>2</sub>	13.8	120	6	89.0	100.0			

\* The amino acid analysis was performed after removal of methylene blue with Norit. Differences in leucine and lysine concentrations were used to correct for protein loss due to adsorption.

according to the method of Henderson and collaborators (19). Tryptophan was estimated by the method of Shaw and McFarlane on the unhydrolyzed protein (20).

Both enzymatic activities decreased progressively during photooxidation (Table V). Maltase activity decreased less than oligo-1,6-glucosidase on a per cent basis. That decreased enzymatic activity was dependent on photooxidation is shown by two types of controls: (1) in the absence of oxygen and in the presence of methylene blue, there was no loss of maltase and only an 11 per cent loss of oligo-1,6-glucosidase activity, (2) in the next to the last experiment (Table V), in the presence of O<sub>2</sub> and absence of methylene blue, after 180 minutes there were a 9 per cent loss of maltase activity and a 12 per cent loss of oligo-1,6-glucosidase activity. In agreement with the findings of Weil and coworkers, histidine was the most

photosensitive amino acid in the intestinal extracts Tyrosine and tryptophan decreased to a much smaller extent than did histidine under these conditions It is to be noted that in these experiments per cent decrease in histidine was greater than per cent decrease in enzymatic activity Similar observations have been made with crystalline lysozyme (21) With other enzymes studied, such as ribonuclease or chymotrypsin (22, 23), per cent decrease in histidine was smaller than per cent decrease in enzymatic activity In the case of lysozyme, since some enzymatic activity remains after complete destruction of histidine, the unoxidized amino acid does not serve an indispensable role in catalysis The present experiments performed with crude extracts indicate that enzymatic activity de-

TABLE VI  
*Inhibition of Intestinal Carbohydrases by Heavy Metals\**

Metal	Oligo-1,6-glucosidase,†	Maltase‡
	<i>per cent inhibition</i>	<i>per cent inhibition</i>
Cu <sup>++</sup>	34	32
Ag <sup>+</sup>	0	44
Hg <sup>+</sup>	0	0
Hg <sup>++</sup>	7 (24)‡	(12)‡
Fe <sup>+++</sup>	0	0
Zn <sup>++</sup>	44	27
Be <sup>++</sup>	0	0

\* The preincubation reaction mixture contained Veronal buffer, 0.06 M, pH 7.95, 0.2 ml, metal salt, 0.004 M, 0.1 ml, enzyme, 0.1 ml, final volume, 0.4 ml

† The reaction mixture contained substrate, 1 mg, preincubation mixture, 0.3 ml, final volume, 2.2 ml It was incubated 30 minutes at 30°

‡ Preincubated 60 minutes instead of 40 minutes

creased upon photooxidation under conditions in which histidine is the most rapidly destroyed of the three photosensitive amino acids tested If prolonged photooxidation of intestinal extracts would lead to complete loss of histidine with some retention of enzymatic activity, similar considerations would apply to the intestinal carbohydrases as apply to lysozyme

#### *Heavy Metal Inhibition*

The effect of preincubation with heavy metals on enzymatic activity was investigated after it was found that recoveries in fractionation were improved in the presence of Versene Typical experiments are presented in Table VI Extracts were preincubated with metal salts (0.001 M) in buffered reaction mixtures for 40 minutes at room temperature Enzy-

matic activity was determined with either maltose or isomaltose as substrate by increase in reducing power. In the concentrations tested, both enzymes were moderately inhibited by  $\text{Cu}^{++}$ ,  $\text{Zn}^{++}$ , and slightly by  $\text{Hg}^{++}$ . Maltase was inhibited by  $\text{Ag}^{+}$ . Inhibition of fungal lactase by mercuric acetate has been observed by Wallenfels and Bernt (14). The enzyme was not inhibited by *p*-chloromercuribenzoate, salyrgan, iodoacetate, or arsenite (14). Smith and coworkers have reported that lysozyme from papaya latex is inhibited by heavy metals and not inhibited by SH reagents (24).

TABLE VII  
*Inhibition of Intestinal Oligo-1,6-glucosidase by Amines\**

pH	Amine	Concentration	Inhibition
		<i>M per l</i>	<i>per cent</i>
7.20	Tris	0.0035	100.0
7.39	"	0.0007	88.6
7.40	"	0.0003	71.8
6.40	"	0.0003	5.0
7.42	Histidine†	0.07	85.5
7.41	"	0.03	52.2
7.41	"	0.014	17.6
7.42	"	0.007	13.3
6.70	"	0.03	5.0

\* The activity was determined as previously described (1), 0.05 ml of enzyme, 60 units, used. The reaction was started by substrate addition.

† L-Histidine monohydrochloride activity was determined as previously described (1), 0.05 ml of enzyme (ammonium sulfate 0.3 to 0.5 saturated fraction), 130 units. The reaction was started by substrate addition.

#### *Amine Inhibition*

Amines, such as histidine, triethanolamine, Tris, have been found to inhibit both enzymes. Inhibitions by histidine and Tris of oligo-1,6-glucosidase, the more sensitive of the two enzymatic activities, are presented in Table VII. Control experiments with hexokinase, glucose-6-phosphate dehydrogenase, and glucose under similar conditions indicated that these enzymes were not inhibited.<sup>7</sup> Two features of the inhibition merit attention. First, the inhibition is pH-dependent. Amines inhibit much more strongly at alkaline than at acid pH. Secondly, inhibition at alkaline pH is reversed in the presence of excess substrate.

The inhibition at alkaline pH suggested that the un-ionized (free base)

<sup>7</sup> Inhibition of oligo-1,6-glucosidase activity by Tris took place in the presence of Versene or Versenol (0.001 M).

form of the amine was the inhibitory species. In further experiments, inhibition at constant pH by a series of four amines of increasing  $pK_a$  has

TABLE VIII  
*Inhibition of Intestinal Carbohydrases by Amines\**

Amine	Inhibition	
	Oligo-1,6-glucosidase†	Maltase‡
	per cent	per cent
Tris	88.0	63.2
2 Amino-2-methyl-1,3-propanediol	52.7	35.5
2 Amino-2-methyl-1-propanol	13.2	6.5
tert Butylamine	0	0

\*The reaction was started by substrate addition

†Amine concentration 0.0007 M, 0.02 ml of enzyme, 30 units, used, pH 7.4

‡Amine concentration 0.001 M, 0.004 ml of enzyme, 30 units, used, pH 7.4

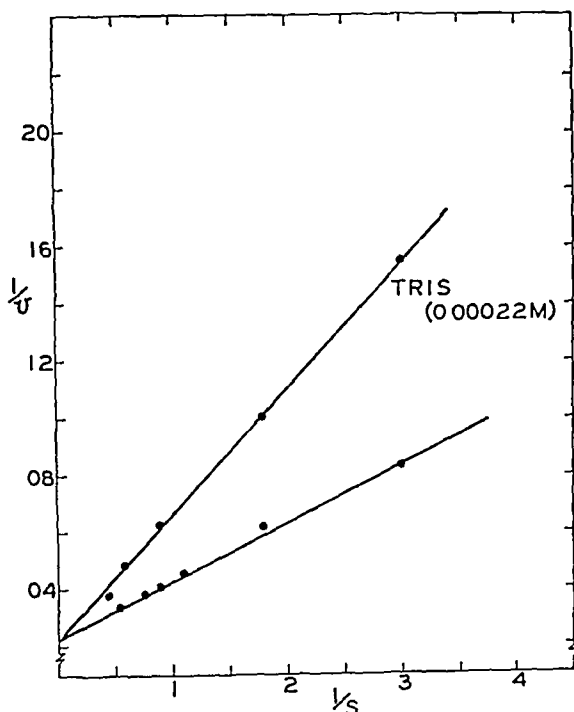


Fig. 4. Inhibition of maltase by Tris. Reaction mixture as in Fig. 2. 66 units of maltase (oligo-1,6-glucosidase-free) used. Reaction started by substrate addition.

as studied (Table VIII). The four amines are of the Tris series in which hydroxyl groups are replaced by methyl groups. The  $pK_a$  of *tert*-butyl-

amine in aqueous solution (0.01 N, 16°) has been determined as 10.8 (2). Titration in aqueous solution gave the following values of  $pK_a$  for the three other amines, uncorrected for ion activities ( $\pm 0.1$  pH unit): Tris, 8.1; 2-amino-2-methyl-1,3-propanediol, 8.9; 2-amino-2-methyl-1-propanol, 9.5. Inhibition of both enzymatic activities decreased with the increasing  $pH$  of the amines. Whether the number of amino hydroxyls is also a contributing factor to the inhibition has not been separately evaluated.

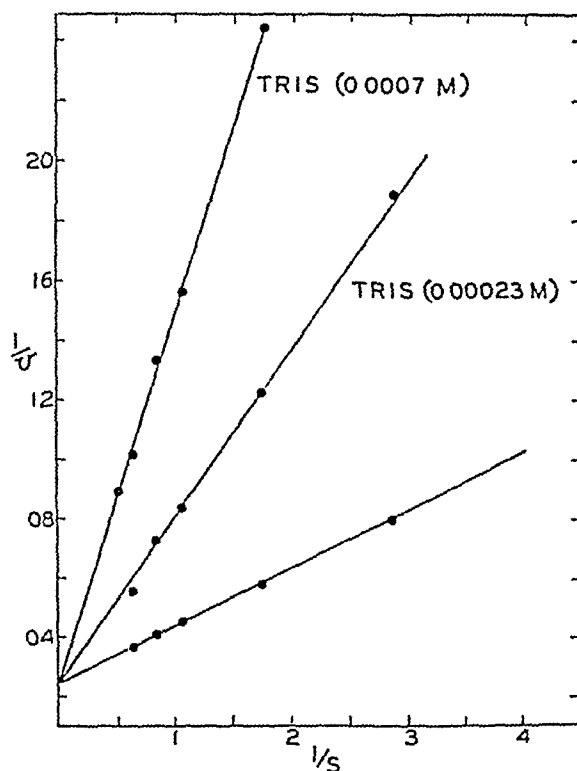


FIG. 5. Inhibition of oligo-1,6-glucosidase by Tris. Reaction mixture as in Fig. 2. 25 units of oligo-1,6-glucosidase used. Reaction started by substrate addition.

similar type of inhibition pattern by this series of amines has been reported by Slein with xylose isomerase (27).

The reversibility of amine inhibition has been further studied with both oligo-1,6-glucosidase and maltase. As shown in Figs. 4 and 5, the Tris-inhibited systems followed the kinetics of reversible inhibition.  $K_i$  values, calculated either on the basis of total amine concentration or on the

\* Titrations were performed at 25°, amine concentrations were as follows: Tris, 6.66 mg. per ml.; 2-amino-2-methyl-1,3-propanediol, 1.01 mg. per ml.; 2-amino-2-methyl-1-propanol, 0.81 mg. per ml. We are indebted to P. Landis of I. H. Kohn Company for these values. Previous  $pK_a$  values recorded for Tris and 2-amino-2-methyl-1,3-propanediol at 23° (0.05 M) are 8.24 and 8.78, respectively (26).

concentration of the free base, are smaller than  $K_m$  values for both enzymes.  $K_i$  values calculated as total amine for maltase and oligo-1,6-glucosidase are  $2.2 \times 10^{-4}$  M and  $1.4 \times 10^{-4}$  M, respectively. When calculated on the basis of the concentration of free base present, assuming a  $pK_a$  of 8.1 for Tris,  $K_i$  values are  $4 \times 10^{-5}$  and  $2.1 \times 10^{-5}$  M, respectively, for the two enzymes.  $K_m$  values in these experiments are  $1 \times 10^{-3}$  M for maltase and  $9 \times 10^{-4}$  M for oligo-1,6-glucosidase. In contrast to these results, Slem has shown that the inhibition of xylose isomerase by Tris does not follow the kinetics of a reversible inhibition (27).

In contrast to chymotrypsin, neither maltase nor oligo-1,6-glucosidase is appreciably inhibited by DFP or TMP. Extracts were preincubated with either agent ( $10^{-3}$  M) for 30 minutes at room temperature in glycylglycine buffer (0.12 M, pH 7.5) and then assayed spectrophotometrically. Inhibitions of the order of 3 to 15 per cent were observed.

#### DISCUSSION

It is of interest to compare amylo-1,6-glucosidase and the intestinal carbohydrases with regard to inhibition by reaction products and by sulfhydryl reagents. Amylo-1,6-glucosidase is inhibited competitively by the polymeric reaction product, the debranched limit dextrin, but not by glucose (3). The intestinal enzymes are inhibited by low concentrations of glucose. With none of these three enzymes is there appreciable incorporation of  $C^{14}$ -labeled glucose into substrate during hydrolysis. In the case of the intestinal enzymes, this holds under conditions by which the enzymes are inhibited by glucose. Product inhibition by hydrolytic enzymes has been frequently noted (28). Amylo-1,6-glucosidase is inhibited by *p*-chloromercuribenzoate and *o*-iodosobenzoate, whereas the intestinal carbohydrases have thus far proved resistant to these reagents.

From the character of the  $pK_m$ -pH plots and the amine inhibitions, it would appear that ionic mechanisms are involved in substrate-binding or overall catalysis. The  $pK_m$ -pH plots indicate that at alkaline pH the enzymes acquire a net negative charge as  $K_m$  increases. It would appear that a protonated (charged) form of the enzyme ( $pK_a$  6.9 to 7.2) rather than the free base is the active species. That such a group may be the imidazole ring of histidine is suggested by its  $pK_a$ , by the decreased enzymatic activity upon photooxidation in the presence of methylene blue, and by the inhibition by heavy metals in the absence of demonstrable inhibition by SH reagents. That other possibilities should be kept in mind is evident. For example, the results of Ryle and collaborators have indicated that the amino groups of cystine in cystyl peptides have unusually low  $pK_a$  values (29).

A possible mechanism which serves as a working hypothesis involving

the imidazole ring of histidine and a secondary enzymatic group in a concerted attack is presented (Fig 6) Fischer-Hirschfelder models reveal that the intermediate eight-membered ring is sterically possible During the stepwise mechanism, the reducing residue is released first, an enzymatic acetal of the original non-reducing residue is formed (presumably with inversion), with the imidazole ring acting both as a hydrogen donor and acceptor Subsequent hydrolysis with the participation of acidic and basic enzymatic groups would release the remaining glucose residue and complete the hydrolysis Amines would be considered as inhibitors by virtue of being bound at the active site It is of interest that aryl amines

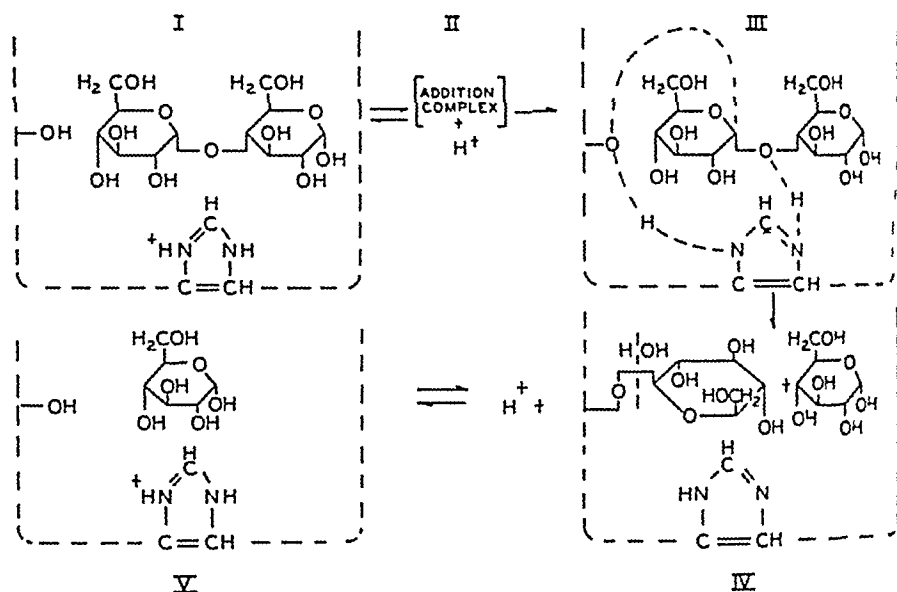


FIG 6 Proposed mechanism of disaccharide hydrolysis III, neutral transition state intermediate

have been found to act catalytically in transglycosidation reactions (30) Stepwise mechanisms involving the intermediate formation of enzyme substrate compounds have been proposed for other hydrolytic enzymes (31)

#### SUMMARY

The properties of the intestinal carbohydrases, maltase ( $\alpha$  glucosidase) and oligo-1,6-glucosidase, have been studied The major fractions of both enzymatic activities are associated with cellular particulates which sediment as microsomes By fractionation of acetone powders, maltase preparations free of demonstrable oligo-1,6-glucosidase activity have been prepared Enzymatic activity is not decreased upon prolonged dialysis against Versene

Enzymes are not inhibited by *p*-chloromercuribenzoate, *o*-iodosobenzoate, iodoacetate, or  $\text{H}_2\text{O}_2$ , but are inhibited by heavy metals such as  $\text{Cu}^{++}$ ,  $\text{Hg}^{++}$ , and  $\text{Zn}^{++}$ . Amines such as tris(hydroxymethyl)amino-methane inhibit at alkaline pH. Inhibition by Tris is competitive with substrate.

pK<sub>m</sub>-pH plots indicate that ionizable enzymatic groups of pK<sub>a</sub> 6.9 to 7.1 are involved in combination with substrate or in over-all enzymatic activity. Enzymatic activities decreased on photooxidation with methylene blue, under conditions in which histidine was the most photosensitive amino acid. A tentative mechanism for hydrolysis involving the imidazole ring of histidine has been proposed.

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# THE SIZE AND SHAPE OF THE RADIOSENSITIVE ACETYLCHOLINESTERASE UNIT\*

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Electric eel organ acetylcholinesterase has been shown by means of ultracentrifuge sedimentation studies to be a very large protein, with a molecular weight of 3,000,000 (2). However, working hypotheses concerning the active center of the enzyme picture this site as being small, *i.e.* comparable in size to the substrate, acetylcholine. This appears to be valid, since assumptions concerning the size and other features of the catalytic surface have been used successfully in predicting the reactivity of various substrates (2, 3), inhibitors (4), and even nerve gas antidotes (5).

From the results of inhibition of eel organ acetylcholinesterase by radioactive diisopropylfluorophosphate, it has been suggested that an enzyme unit much smaller than the molecule itself does exist (6). In order to demonstrate and to describe in a more precise manner submolecular units of this enzyme, we applied the techniques of Lea (7) and of Pollard *et al.* (8), utilizing inactivation by ionizing radiation to study the relationship between molecular organization and enzymatic activity.

Preparations of eel organ acetylcholinesterase were subjected to irradiation by cobalt-60  $\gamma$ -rays, electrons, protons, and  $\alpha$  particles. From analyses of the data of the resulting inactivation we have postulated the existence of a small, radiosensitive acetylcholinesterase unit and have derived indications as to its size and shape.

## Materials and Methods

*Irradiation of Dilute Enzyme Preparations*—Dilute preparations of a lyophilized, partially purified extract<sup>1</sup> of electric eel organ tissue were prepared in potassium phosphate buffer, pH 7.2,  $\mu$  0.1 (1.29 gm of  $\text{KH}_2\text{PO}_4$ , 5.25 gm of  $\text{K}_2\text{HPO}_4$  per liter, Baker and Adamson). Samples were placed in cork-stoppered Lucite tubes, which were then sealed with tape and irradiated in a hollow, cylindrical, cobalt-60  $\gamma$  source at 200,000 roentgens per hour.

\* A preliminary report of this work has been presented (1). This research was supported by the United States Atomic Energy Commission.

<sup>1</sup> Kindly supplied by Dr. I. B. Wilson and Dr. M. Altamirano, respectively, Department of Neurology, College of Physicians and Surgeons, Columbia University.

*Irradiation of Dried Enzyme Preparations*—Tissue fluid from presorted homogenized segments of frozen electric eel organ tissue<sup>1</sup> was treated at  $110,000 \times g$  for 30 minutes in the Spinco preparatory centrifuge. 0.05 ml aliquots of the clear supernatant fraction were dried by a vacuum at room temperature on circular glass cover slips. Samples, including controls, were held with "Lubriscal" stopcock grease on an aluminum bombardment disk, which was then placed in one of two irradiation chambers according to the charged particle source.

After the appropriate chamber was connected to the vacuum of the Brookhaven 60 inch cyclotron, the dried enzyme was subjected to a monoenergetic, collimated beam of either 40 m.e.v.  $\alpha$  particles or 10 m.e.v. protons. By rotation of the bombardment disk through an O ring seal the various samples were exposed to the beam.  $\alpha$  particles of lower energies were obtained on occasion by covering the enzyme samples with aluminum foils of measured thickness.<sup>2</sup> Other samples were irradiated with 2 m.e.v. electrons (only roughly collimated in the chamber used) from the Van de Graaff generator in the Brookhaven National Laboratory. In the Chemistry Department, the particles entering the evacuated chamber through a thin aluminum window.

The experimental arrangement for the various bombardments provided for collection of all the particles incident on an area larger than the sample being irradiated. The beam, which uniformly covered the irradiated area, was measured as an electric charge by a current integrator.

After bombardment, in all cases at room temperature, the cover slip were placed in potassium phosphate buffer, the appropriate dilutions were made, and the remaining enzymatic activities were determined by a microdiffusion procedure for acetic acid (9). The per cent activities remaining were then plotted as ordinates on semilogarithm paper in relation to the doses delivered.

## RESULTS AND DISCUSSION

*Irradiation in Wet State*—An indication that eel acetylcholinesterase activity may not be dependent on the integrity of the entire molecule was determined from a study of the heat sensitivity of the enzyme irradiated in a dilute state. An aliquot of a preparation of 274  $\gamma$  of lyophilized tissue extract per ml of phosphate buffer was irradiated with cobalt-60  $\gamma$  rays until only 2 per cent activity remained. The residual enzyme as well as solutions of the non-irradiated control (both diluted 1:10 with buffer) were heated at 47.8° for the times indicated in Fig. 1. The heat sensitivity

<sup>2</sup> End ranges of  $\alpha$  particles were determined by using bacteriophage T1 inactivation as an indicator.

of the irradiated enzyme was greater than that of the control, implying that the molecules surviving the irradiation had been partially damaged. A possible interpretation was that the molecule as a whole was not essential to the enzymatic activity.

*Irradiation in Dry State*—In order to use ionizing radiations to study the molecular organization of eel organ acetylcholinesterase, it was necessary to irradiate dried enzyme preparations.

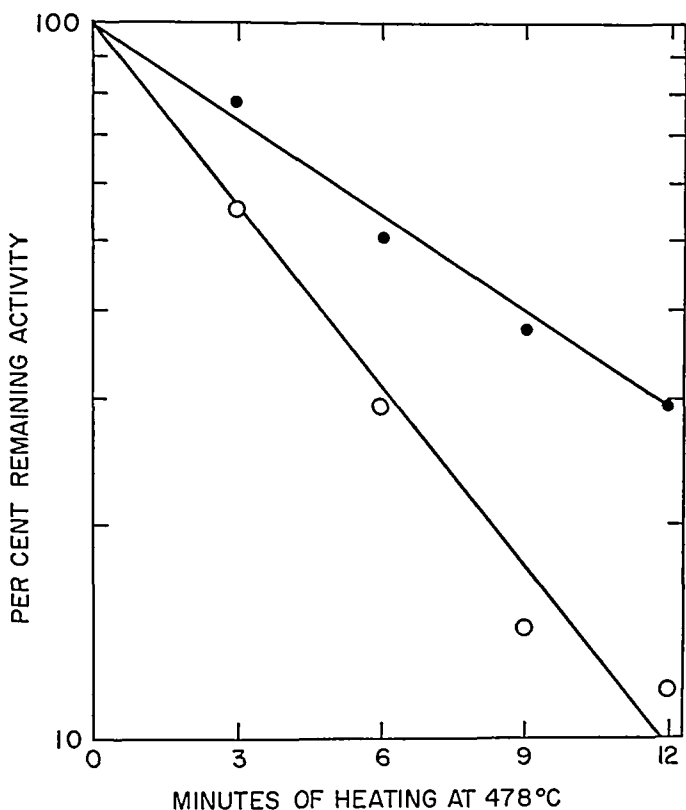


FIG 1 Heat inactivation at 47.8° of a dilute preparation of eel acetylcholinesterase irradiated in the wet state to 2 per cent of the original activity (○) relative to the non-irradiated control (●)

Lea (7) and later Pollard *et al* (8) interpreted the loss of activity of dried biological materials subjected to ionizing radiations in terms of radiosensitive volumes associated with the active principles. The method is independent of the purity of the irradiated preparations. With a number of exceptions, reasonable agreement has been found between sizes of radiosensitive units and the molecular weights determined by physicochemical methods for many active materials which range in size from viruses to antibiotics.

*Basic Principles Involved in Calculating Target Sizes*—Inactivation of dried biological materials by ionizing radiation is attributed to alterations in structure which result from ionizations of atoms of the target molecule. A radiosensitive unit may be defined as the aggregate of atoms with which one primary ionization event results in loss of biological activity.

Since inactivation has been found in these experiments to be exponentially related to the radiation dose, the proportion of material still active after a given dose,  $I$ , is given by

$$n/n_0 = e^{-VI}$$

When  $I$  is expressed as the number of primary ionizations occurring per ml (or gm) of sample,  $V$  is the volume (or mass) of the radiosensitive unit (8). In order to determine  $V$ , it is convenient to know the dose at which only 37 per cent of the biological activity remains.  $V$  is then equal to the reciprocal of  $I$ , since  $e^{-1} = 0.37$ .

*Cobalt-60  $\gamma$  Radiation*—In the course of utilizing cobalt-60  $\gamma$  radiation to resolve mixtures of acetylcholinesterase into sedimentable and non-sedimentable states,<sup>3</sup> dried films of partially purified tissue extracts and lyophilized segments of whole tissue were found to give exponential rate of inactivation after irradiation. From the rates, which were identical, it was calculated that  $4.8 \times 10^6$  roentgens lowered the enzymatic activity to 37 per cent of that observed prior to irradiation.

Although cobalt-60  $\gamma$ -rays yield primary ionization events essentially randomly distributed through the irradiated material, some clustering does occur. Lea has calculated corrections for such overlapping in terms of his "associated volume method." On Lea's graphical presentation of this method (7) a 37 per cent survival dose of  $4.8 \times 10^6$  r corresponds to a target molecular weight of 170,000.

More explicitly, with the assumption that primary ionization events are randomly distributed, the number of events occurring per gm of sample may be determined by dividing the radiation dose, converted to electron volts per gm, by 110 e v, the average energy lost per primary ionization (p1). The reciprocal, multiplied by Avogadro's number, is the target molecular weight.

$$(6.03 \times 10^3) \times \frac{(1.6 \times 10^{-12}) \text{ ergs per e v} \times 110 \text{ e v per p1}}{93 \text{ ergs per gm per r} \times (4.8 \times 10^6) \text{ r}} = 237,000 \text{ gm per r p1}$$

Thus the weight of the radiosensitive unit of cell acetylcholinesterase is

<sup>3</sup> Serlin, I., and Cotzias, G. C., paper accepted for publication by *Radiation and Life* search.

determined by either method for analyzing  $\gamma$ -radiation data, was roughly one-fifteenth that of the macromolecule

As Pollard (10), however, has pointed out, in irradiation with  $\gamma$ -rays "a wide mixture of forms of ionization are [is] present" In order, therefore, to make the size of the radiosensitive unit less equivocal, it was necessary to turn to the use of charged particles

*Principles of Calculating Target Sizes from Charged Particle Inactivation Data (8)*—As charged particles move through target matter, they transfer energy along their paths at a rate determined primarily by their charge

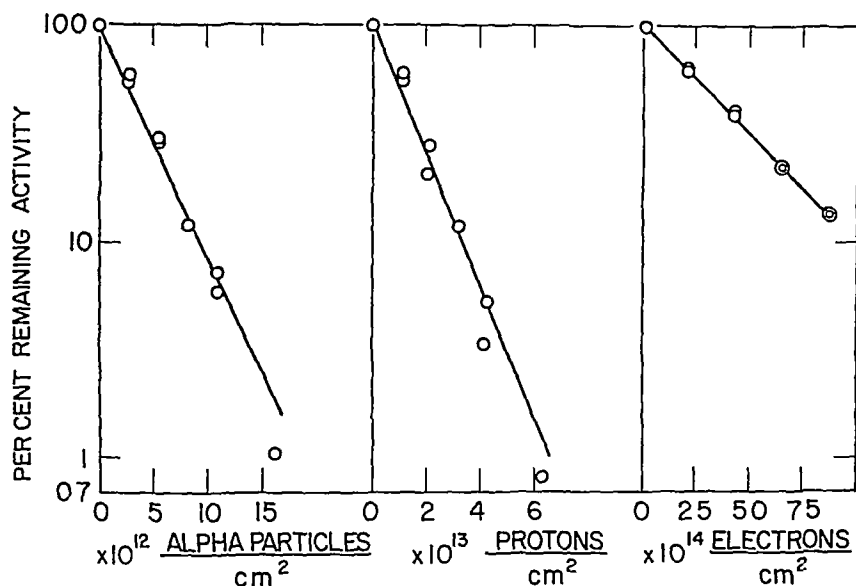


FIG 2 Inactivation of dried films of eel acetylcholinesterase by 40 m e v  $\alpha$  particles, 10 m e v protons, and 2 m e v electrons

and velocity For example, a 40 m e v  $\alpha$  particle has a linear energy transfer (LET) of 240 e v per 100 A of protein equivalent material LET values were calculated from the Bethe-Block equation (11) with a protein composition of  $C_4H_8NO_2$  Since 110 e v are transferred per primary ionization, this energy loss can be translated into the number of primary ionizations occurring along the particle path

In addition to the rate of energy loss, the area covered by the particle beam must also be determined This allows the dose to be expressed in terms of the number of particles delivered per unit of area Since charged particle inactivation of dried acetylcholinesterase is also exponential in relation to dose (cf Figs 2 and 3), the reciprocal of the 37 per cent dose in this treatment is an inactivation cross section or radiosensitive area,  $S$  This cross section is a function of LET Sparsely ionizing particles (parti-

cles with low LET values) can pass through enzyme molecules with small probability of the occurrence of ionization, thus indicating a small radio-sensitive area. For densely ionizing particles,  $S$  may be correlated with the maximal cross sectional area for inactivation,  $S_0$ . Since primary ionization events are randomly distributed along particle tracks, the relation between

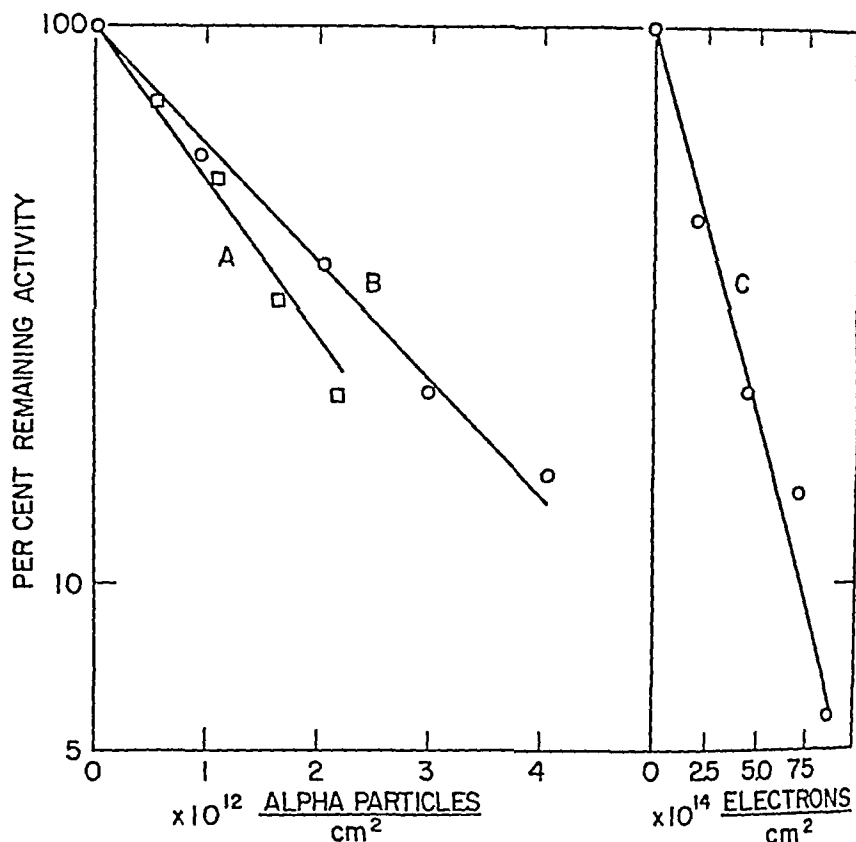


FIG 3 Inactivation of dried films of eel acetylcholinesterase by charged particles which were passed through aluminum foil. Curve A, 6 m e v  $\alpha$  particles, Curve B, 10.7 m e v  $\alpha$  particles, Curve C, 2 m e v electrons (passed through 40 mil foil)

$S$  and LET may be described by the equation

$$S = S_0[1 - e^{-(t \times (LET/110 \text{ e v per p l}))}] \quad (1)$$

The term in brackets presents the chance that at least one primary ionization occurs within a target of thickness  $t$  and area  $S_0$ . If LET is expressed in electron volts per cm,  $t$  is in cm per p l.

For irradiation at low LET values, the exponent in Equation 1 is  $\ll 1$ , and an approximate formula may be substituted

$$S = S_0 t \times \frac{LET}{110 \text{ e v per p l}} \quad \text{or} \quad S_0 t = S \times \frac{110 \text{ e v per p l}}{LET}$$

Since  $S_0$  is the radiosensitive volume, approximate target molecular weights may be calculated at low LET values as follows

$$\text{Mol wt} = S(\text{sq cm}) \times \frac{1}{\text{LET (e v per cm)}} \times 110 \text{ e v per p} \times \quad (2)$$

$$1.35 \text{ gm per ml} \times 6.03 \times 10^{23}$$

The density figure for protein, 1.35 gm per ml, was also used in calculating

TABLE I  
*Eel Acetylcholinesterase 37 Per Cent Survival Doses after Irradiation  
with Charged Particles\**

Particle	Energy of particle	Linear energy transfer	37 per cent dose particles per sq cm	Target mol wt
	<i>mev</i>	<i>ev per 100 A</i>		
Electron	2	2.53	$5.0 \times 10^{14}$	71,000
"	2	2.53	$5.0 \times 10^{14}$	71,000
" †	2	2.53	$3.5 \times 10^{14}$	100,000
Proton	10	61	$1.3 \times 10^{13}$	110,000
"	10	61	$1.5 \times 10^{13}$	95,000
$\alpha^\ddagger$	40.0	240	$4.5 \times 10^{12}$	83,000
$\alpha$	40.0	240	$4.2 \times 10^{12}$	89,000
$\alpha$	40.0	240	$3.9 \times 10^{12}$	95,000
$\alpha^\ddagger^\ddagger$	28.5	320	$4.1 \times 10^{12}$	
$\alpha^\ddagger^\ddagger$	16.2	500	$2.7 \times 10^{12}$	
$\alpha^\ddagger$	16.2	500	$2.2 \times 10^{12}$	
$\alpha^\ddagger$	10.7	690	$2.0 \times 10^{12}$	
$\alpha^\ddagger^\ddagger$	9.5	760	$1.9 \times 10^{12}$	
$\alpha^\ddagger^\ddagger$	7.4	910	$1.7 \times 10^{12}$	
$\alpha^\ddagger^\ddagger$	6.5	1020	$1.7 \times 10^{12}$	
$\alpha^\ddagger$	6.0	1050	$1.5 \times 10^{12}$	

\* 37 per cent doses were rounded off to two significant figures

† Aluminum foils were placed between the sample and the beam

‡ The 37 per cent dose was calculated on the basis of single point irradiations

LET values, and as a result the molecular weight values are independent of this figure

*Charged Particle Inactivation of Eel Organ Acetylcholinesterase*—The inactivation of eel organ acetylcholinesterase by  $\alpha$  particle, proton, and electron bombardment was found in all cases to be exponentially related to the dose (cf Fig 2).  $\alpha$  beams with higher LET values than 240 e v per 100 A were obtained by placing aluminum foils of varying thickness between the beam and the sample. In these experiments the values for the energy, and hence LET, of the resulting beams were calculated upon the determination of the thickness of aluminum foil which could fully



stop the particles.<sup>2</sup> These higher LET  $\alpha$  particles also yielded exponential inactivations (cf Fig 3, Curves A and B).

After the fact was established that the inactivation of eel organ acetylcholinesterase was logarithmically related to dose over a wide range of LET, single point irradiations at various LET values were also performed. These allowed 37 per cent survival doses to be determined from assumed exponential relationships. In Table I are listed the 37 per cent survival doses from each of the exponential survival curves, together with the

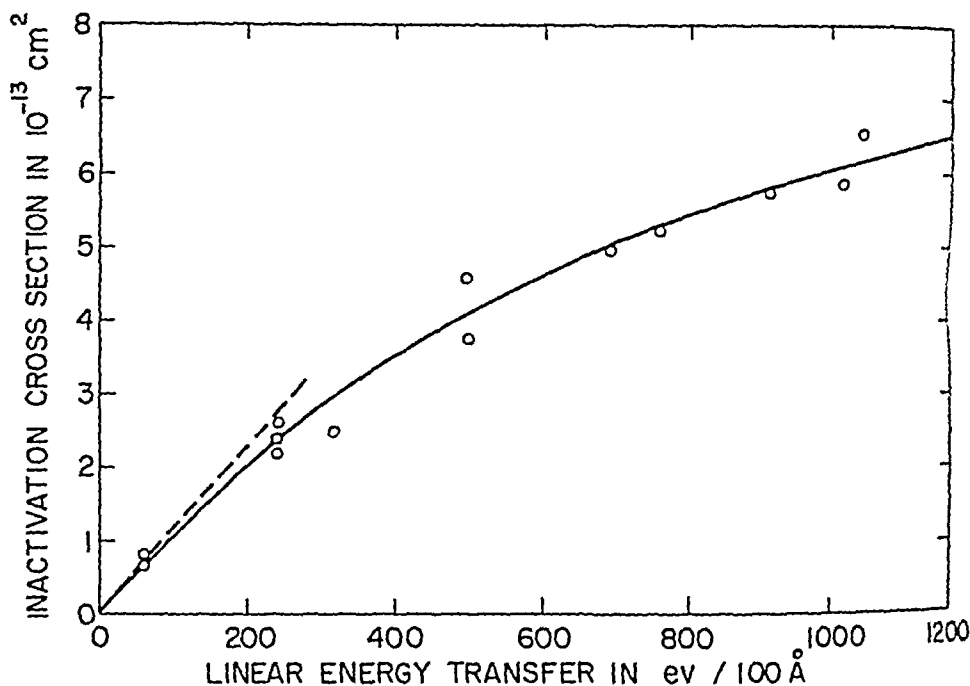


FIG 4 Inactivation cross section of eel acetylcholinesterase as a function of the rate of energy loss by charged particles in protein matter. The slope at the origin represents electron data (obtained from Fig 3, Curve C).

corresponding energies and LET values of the various charged particles. Also given in Table I are approximate target molecular weights calculated from Equation 2, with only those data obtained at lower LET values.

Values for the radiosensitive area  $S$  are shown in relation to linear energy transfer in Fig 4. The smooth curve is a plot of Equation 1 fitted to the data of Table I as follows:

$$S = 7.8 \times 10^{-13} \text{ sq cm} [1 - e^{-(16.5 \times 10^{-3} \text{ cm per p.l.} \times (\text{LET e.v. per cm} / 110 \text{ e.v. per p.l.}))}]$$

The radiosensitive volume,  $S \cdot t$ , thus appears to be an area of about  $7800 \text{ Å}^2$  times an average thickness of approximately  $16.5 \text{ Å}$ . The volume, multiplied by the density figure,  $1.35 \text{ gm per ml}$ , and by Avogadro's number, gives a final target molecular weight of  $105,000$ .

The electron inactivation data, shown in Fig 4 as a slope at the origin, were from an experiment in which a 40 mil thickness of aluminum was placed over the samples (cf Fig 3, Curve C) The aluminum was intended to compensate for escape of energetic secondary electrons from the thin layers of enzyme, a possibility for 2 mev electron bombardment However, we have not ruled out other explanations for the agreement among these and the other charged particle data

The target cross section and thickness are incompatible with a spherical radiosensitive unit A shape for the target volume cannot be unambiguously determined, for example, either a thin cylinder or a flat plate could be fitted to the data If the target unit is assumed to be a cylinder lying transverse to the beam, a diameter of 21 A and a length of 360 A can be deduced

By way of comparison with the radiosensitive molecular weight of 105,000, the equivalent weight of the enzyme, as determined by diisopropylfluorophosphate-binding studies and based on the most active preparation reported (2), has been calculated to be 63,000 (6) In view of the macromolecular size of 3,000,000, the 2 submolecular units are of such sufficiently similar magnitude as to indicate a possible identity If true, this would serve to demonstrate (a) that the most active preparation known (2) consisted of essentially pure enzyme, and (b) that electric eel organ acetylcholinesterase is a molecular aggregation of 30 to 50 enzymatically active units

A more extensive cyclotron investigation is tentatively planned to compare the radiosensitive volumes of acetylcholinesterase from different sources within single species Preliminary studies with the use of cobalt-60  $\gamma$ -radiation (1) have indicated a variation in target size, according to the tissue source

#### SUMMARY

1 Thin, dried films of electric eel organ acetylcholinesterase have been exposed to electron, proton, and  $\alpha$  particle bombardment

2 Rates of inactivation were found to be related exponentially to the doses delivered

3 The radiosensitive unit of the enzyme has been indicated to have a molecular weight of 105,000 and to be non-spherical On the assumption of a cylindrical shape, the unit was found to be 360 A long and 21 A in diameter

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# EXPERIMENTAL INJURY AND SULFUR AMINO ACID METABOLISM\*

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It has been reported that methionine and cystine are capable of accelerating the healing rate of wounds in injured rats to the same extent when present in the diet in equivalent amounts (1). This response was elicited in both the presence and the absence of dietary protein (2). Lysine, tryptophan, and valine do not appear to alter the healing process to a significant degree (3). Because methionine is converted to cystine, although the reverse reaction does not occur to an appreciable extent *in vivo* (4, 5), it was suggested that cystine might be the more limiting amino acid for the healing of experimental wounds (1). This supposition appears to have been substantiated from analyses of wounded and corresponding unwounded skin tissue. When healing is thought to be essentially complete, *i.e.* when the nitrogen content of wounded and corresponding normal tissue is similar (6), the cystine level in the former is 2.5 times that of the normal tissue, and the methionine content has also increased, but to a lesser degree (2).

The excessive demand for the sulfur-containing amino acids by regenerating wound tissue, along with the negative nitrogen balance characteristic of injury, suggests rather strongly that the phenomena of wounding and regeneration markedly influence protein metabolism. A previous report dealt with the incorporation of  $S^{35}$ -labeled methionine and cystine into various tissues after experimental injury (2). The effect of injury on sulfur amino acid metabolism will be considered in the present paper.

## EXPERIMENTAL

In this study, 80 female albino rats of average weight,  $200 \pm 20$  gm, were used, and the animals were maintained on a protein-free ration for a period of 4 days. The composition of the diet is presented elsewhere (2). Each animal was offered 8 gm of food daily, which was completely consumed, and distilled water was permitted *ad libitum*. At the termination of the 4 day acclimation period,  $S^{35}$ -labeled DL-methionine ( $4.0 \times 10^5$  cpm)

\* This investigation was supported in part by a grant from the North Dakota Cancer Society.

was administered intraperitoneally to each rat 48 hours later the animals were anesthetized with sodium pentobarbital. Half of the animals were wounded by inflicting a standard 3 cm circular skin wound on the back of the neck in the area of the shoulder blades (2), the remaining animals served as controls. Eight rats from each group were again anesthetized at various times after the administration of the tracer amino acid and samples of tissue from wound, muscle, and liver were excised, weighed, and hydrolyzed in a mixture of 20 per cent HCl-50 per cent HCOOH for 24 hours (7). Aliquots of the hydrolysate were then analyzed for methionine (8) and cystine plus cysteine (referred to as *cystine*) (9). Total  $S^{35}$  was determined by adding carrier sulfate and precipitating the sulfate as barium sulfate after oxidation with  $HNO_3$ - $HClO_4$  (10). The  $S^{35}$ -labeled *cystine* was precipitated as the cuprous mercaptide (11) and treated as outlined for total  $S^{35}$ . Methionine- $S^{35}$  was calculated by difference. The activities, corrected for decay, were determined at "infinite thickness" with a windowless gas flow counter.

#### RESULTS AND DISCUSSION

The present work was undertaken to determine whether experimental injury causes alterations in the metabolism and distribution of the sulfur-containing amino acids. Data from a previous report suggested that wounding influences the incorporation and distribution of  $S^{35}$ -labeled methionine and *cystine* when DL-methionine- $S^{35}$  was administered after injury (2). In the present study the animals were furnished labeled methionine- $S^{35}$  and subsequently wounded. This procedure permitted a clearer insight into the effect of experimental injury on the metabolism of methionine and *cystine*.

In Table I are shown the specific activities of methionine and *cystine* in regenerating wound tissue. The term "specific activity" is defined as (counts per minute per gm of tissue) (body weight) (100)/(mg amino acid S per gm of tissue) (dose). It can be seen that the turnover rate for the sulfur amino acids in tissue from wounds is relatively high, the half life being approximately 5 days in the case of methionine for animals maintained on a protein-free diet. These data are commensurate with previously reported results and probably reflect a high rate of protein synthesis (2).

Fig 1 represents a graph of the specific activity of methionine- $S^{35}$  plotted against time for muscle and liver tissue from injured and unwounded rats. There appears to be a marked increase in the total liver methionine pool directly after wounding. Coincident with this alteration, the total extrahepatic methionine pool, as reflected in muscle, diminishes. These data seem to indicate that wounding causes a mobilization of methionine in

extrahepatic sources for use by the liver of the injured animal. Furthermore, 4 days after wounding, the total liver methionine pool in the wounded rat decreases rapidly, with a concomitant shortening of the total liver methionine half life. This latter effect may mean that there is an increase

TABLE I  
*Specific Activity of Methionine-S<sup>35</sup> and Cystine-S<sup>35</sup> in Wound Tissue after DL-Methionine-S<sup>35</sup> Administration*

Days after wounding	Methionine S <sup>35</sup>	Cystine S <sup>35</sup>
4	10.05 ± 1.18*	7.51 ± 0.14
7	5.72 ± 0.35	5.58 ± 0.56
10	4.50 ± 0.42	6.45 ± 0.58
13	3.89 ± 0.37	3.66 ± 0.34

\* Standard deviation of the mean

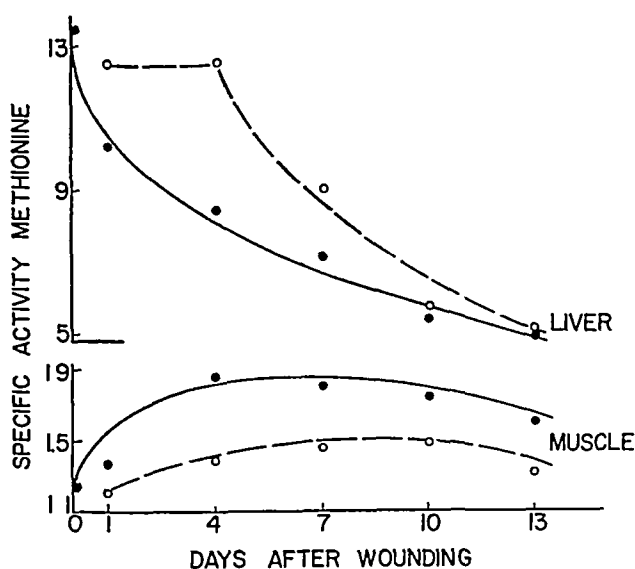


Fig. 1 Specific activity of methionine in liver and muscle tissue from wounded (open circles) and unwounded control animals (solid circles). DL-Methionine-S<sup>35</sup> was administered 48 hours before Day 0.

in the degradative rate of methionine metabolism in the liver after injury. The initial increased specific activity in liver tissue after experimental injury may be explained in the following manner. It might be expected that a mobilization of methionine for the liver would produce little alteration in the specific activity either in the liver or at the source. However, the data can be reconciled if it is assumed that the injected DL-methionine-S<sup>35</sup> is incorporated rapidly into the "labile" protein (12) extrahepatic

tissue, is incorporated at a slower rate into the structural protein fraction, and is mobilized from the former after wounding.

The plot in Fig. 2 depicts the specific activity of *cystine* from muscle and liver tissue after administration of  $S^{35}$ -labeled DL-methionine from the wounded and control animals. The experimental injury apparently causes a diminution in the size of the total liver *cystine* pool. This alteration may be attributed either to an increased rate of *cystine* degradation or loss, or to a decreased synthesis from methionine. The apparent lowering of the total liver *cystine* pool after injury, in the presence of an increase in the

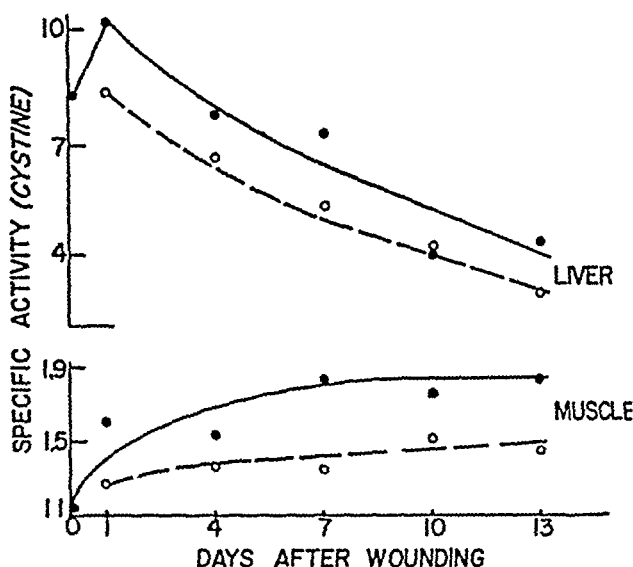


FIG. 2 Specific activity of *cystine* in liver and muscle tissue from wounded (open circles) and unwounded control animals (closed circles). DL-Methionine  $S^{35}$  was administered 48 hours before Day 0.

size of the total liver methionine pool, may mean that there is a loss of liver *cystine* after wounding, rather than a decreased formation of *cystine*. This conclusion appears to be supported by previous investigations, which suggest that injury increases the rate of methionine conversion to *cystine* (13). Furthermore, it has been demonstrated that liver is one of the chief sites of *cystine* formation from methionine (14). Thus, it might be possible to conclude that, after wounding, there is a mobilization of methionine for the liver of the injured rat. This amino acid may then be converted to *cystine* to meet the requirements of the regenerating wound tissue for *cystine*.

The data in Fig. 2 suggest that experimental injury causes a decrease in the size of the muscle or extrahepatic total *cystine* pool. This may be due to a loss of *cystine* from extrahepatic tissue to supply the healing wound.

with *cystine*, to a decreased availability of methionine, or a combination of both effects

It has been reported previously that, although the total amount of sulfur excreted by the normal and wounded rat remains essentially the same, there is an increase in the excretion of sulfate, with a concomitant decrease in the excretion of non-sulfate sulfur by the injured animal (13). Because the oxidation of *cystine* to sulfate occurs primarily in liver (15), it may be possible to advance an explanation from the present data for the increased formation of sulfate after wounding. It might be supposed that an increased concentration of substrate, in this case methionine and *cystine*, in liver tissue would result in an increased formation of sulfate.

#### SUMMARY

Experimental injury causes a mobilization of methionine from muscle for use by the liver of the wounded rat. Methionine may then be converted to *cystine* in the liver to meet the *cystine* requirement of the regenerating wound. The data indicate that injury produces a decreased specific activity with respect to *cystine* in muscle tissue, however, the significance of this alteration remains obscure.

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# OCCURRENCE AND NATURE OF A FECAL PHOSPHORUS-CONTAINING LIPIDE\*

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There is little information in the literature regarding the occurrence and nature of fecal lipide phosphorus. Recently, Kim and Ivy (1) reported that rats fed a fat-free diet excreted only a trace of lipide phosphorus in their feces. When corn oil was added to a fat-free diet, appreciable amounts of lipide phosphorus appeared in the feces, and the addition of oleic acid or fatty acids of corn oil increased the lipide phosphorus excretion further. Palmitic acid had only a mild stimulatory action on lipide phosphorus excretion. Pihl (2) confirmed these findings in animals fed oleic acid, and also observed that, when 4 volumes of acetone were added to an ether solution of the fecal lipides, a white precipitate, which later changed to brown, was formed. The precipitate could be redissolved in ether. All of the authors mentioned above referred to the material as phospholipide, since it precipitated with acetone and contained phosphorus.

In the course of investigations on the absorption of plant sterols in these laboratories (3), several methods were tried for complete extraction of fecal lipides. It was observed that hot acetone-alcohol (1:1) extracts of feces developed a white precipitate on cooling which later became brown. Extraction with 2:1 chloroform-methanol and washing with water according to Folch *et al* (4) gave complete extraction of the lipides and a solution which did not deposit solid material on cooling. After determination of the fecal sterols, the remainder was analyzed for lipide P, with the results shown in Table I. The fat-free diet resulted in only a trace of lipide phosphorus in the feces (0.07 mg per day). With the addition of 5 per cent oleic acid to the diet, the lipide P increased to 1.32 mg per day, while, at the 25 per cent level, 10.94 mg of lipide P per day were excreted. The addition of bile salts to the diet of oleic acid diminished lipide P excretion to 4.60 mg per day.

The chloroform extracts were treated with 4 volumes of acetone and a few drops of saturated  $MgCl_2$  in methanol. The precipitate in the moist

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state<sup>1</sup> was readily soluble in chloroform. The substance was precipitated three times from chloroform solution by acetone and then dissolved in chloroform, and the material was subjected to analysis. Lipide P was found to be 2.3 per cent, there was only a trace of nitrogen. The material was soluble in chloroform, ether, petroleum ether, and benzene when moist,<sup>1</sup> and insoluble in water, acetone, methanol, and ethanol. It could be precipitated from chloroform or ether solution by acetone or methanol. The fatty acid content of the material which was precipitated four times was 75 per cent. Further qualitative tests were carried out on the original material and on the hydrolysates, and none of the following could be detected: glycerol (5), choline (6), carbohydrate by the anthrone test, sodium, and potassium. These tests were repeated a number of times on several different samples with the same results. Attempts were made to

TABLE I  
*Effect of Oleic Acid on Fecal Excretion of Phosphorus-Containing Lipide from Seven Rats*

Addition to diet	Lipide P*
	mg per day
Fat-free	0.07
" + 1% sodium taurocholate	0.07
5% oleic acid	1.32
25% " "	10.91
25% " " + 1% sodium taurocholate	4.60

\* Average excretion per day for a 7 day period

purify the material further by removing adhering fecal pigment by shaking a chloroform solution with cold 1 N HCl for 10 minutes. It was then observed that the material no longer gave a precipitate with methanol or acetone, and the aqueous phase contained all the phosphorus as inorganic phosphate. The chloroform phase contained all of the fatty acid, which had an iodine number of 84. These preliminary experiments indicated that the material was not a true phospholipide, therefore it was decided to conduct further experiments on the nature of this substance. Rats were fed a diet high in oleic acid, and the lipide P-containing material was isolated and studied, P<sup>32</sup> being used as a tracer.

### *Materials and Methods*

*Animals and Diet*—Rats, weighing 175 to 200 gm., were housed in individual cages arranged for quantitative collection of feces. The di-

<sup>1</sup> After drying to constant weight at room temperature under a vacuum, the material was insoluble in all solvents.

in slight excess of the daily requirement (10 gm) was weighed and placed in food cups, and the diet remaining the following day was weighed and the food intake calculated by difference. To the diet were added  $20 \mu\text{c}$  of  $\text{P}^{32}$  each day for a total of  $140 \mu\text{c}$  per week per rat. The actual  $\text{P}^{32}$  intake was calculated from the data on the food intake. The diet consisted of 20 per cent casein, 24 per cent corn starch, 24 per cent glucose, 25 per cent oleic acid, 2 per cent methyl cellulose, 5 per cent Hubbell-Mendel-Wakeman salt mixture, and adequate amounts of the crystalline vitamins. The diets were fed for 7 days. Feces were collected daily, pooled, and refrigerated.

In a short term experiment eight rats were divided into two groups. In one group each rat received  $0.5 \mu\text{c}$  of  $\text{P}^{32}$  per gm of body weight intraperitoneally plus the oleic acid diet, the rats in the other group received the same amount of  $\text{P}^{32}$  in the oleic acid diet. The rats were killed after 24 hours, and the feces and intestinal contents were collected and pooled for each group.

*Treatment of Feces*—The pooled feces were ground and extracted four times with boiling 2:1 chloroform-methanol. The extract was washed with water, dried over  $\text{Na}_2\text{SO}_4$ , and filtered, and the volume was determined (2300 ml) and a sample taken. The extract was concentrated to 250 ml in an atmosphere of nitrogen. The concentrated chloroform extract was divided in half. One-half was precipitated eight times by acetone and the other half seven times by methanol. Samples were taken at each precipitation for analysis and  $\text{P}^{32}$  counting.

In the short term experiment, the feces were ground and added to the intestinal contents. Extraction and concentration were carried out as for the previous group, one precipitation by acetone and four precipitations by methanol being carried out.

*Analysis*—The chloroform extracts of each precipitation were analyzed for total phosphorus by acid digestion, followed by the Fiske-Subbarow color reaction (7). Total ash was determined by heating at  $550^\circ$  overnight and by weighing the residue. A sample weight was determined by drying an aliquot of the chloroform extract to constant weight, and the total calcium was determined by treating an aliquot of the extract with 5 N  $\text{H}_2\text{SO}_4$ , heating until dry, and ashing at  $550^\circ$ . The ash was dissolved in HCl and calcium was determined by titration of the oxalate with  $\text{KMnO}_4$ .

The fatty acid content was determined by shaking at room temperature an aliquot of the chloroform solution with 1 N HCl for 10 minutes. The chloroform solution, containing the fatty acids, was washed with water, dried over sodium sulfate, and filtered. The solvent was removed and the fatty acids were weighed and then titrated with standard alcoholic KOH to determine the average molecular weight. Iodine numbers were also

determined on the fatty acids. The aqueous phase from the acid treatment was analyzed for inorganic phosphate and calcium.

The  $P^{32}$  content was determined at each precipitation, corrected for decay, and the activity expressed in terms of counts per minute per microgram of P and counts per minute per mg of lipid.

### Results

The data on the effect of methanol precipitation on the composition of the lipid are shown in Table II. The phosphorus content of the lipid increased from 1.52 to 4.21 per cent, a 2.8-fold increase. All of the phosphate at each precipitation was recovered in the acid hydrolysate. Calcium formed the same pattern and increased from 6.7 to 11.8 per cent, a 2-fold increase. Concomitantly, the fatty acid content decreased from 83.5 to 69.9 per cent, this decrease being approximately the same as the increase in the inorganic fraction. The percentage accounted for is approximately 100 per cent as the ash plus the fatty acid, and about 100 per cent when the individual fractions were totaled, indicating a small amount of inorganic material not accounted for in the analysis. In the latter figure, P was calculated as  $PO_4$ .

The counts per minute per microgram of P were about the same throughout the precipitations, indicating that virtually all of the  $PO_4$  was in the lipid. On successive precipitations all of the  $P^{32}$  could be recovered in the precipitate. Calculation of the amount of total lipid in the original chloroform extract showed that over 85 per cent of the total fecal lipid was present as the phosphorus-containing lipid. The counts per minute per mg of lipid increased as the compound was purified, indicating approximately a 2-fold purification. Of the total amount of  $P^{32}$  consumed 26.4 per cent was recovered in the lipid.

Precipitation was also carried out eight times by acetone. Methanol treatment gave a higher P content than acetone, and the data are not included, since they demonstrate similar increases in P and Ca and a drop in fatty acid content. In further experiments, as many as eleven precipitations by methanol were carried out. The fatty acid continued to decrease, while  $PO_4$  and Ca increased.

The previous experiments had suggested that the lipid was not a true phospholipide, but probably a salt. In order to obtain more information regarding its salt-like nature, the pK of the compound was determined. A chloroform solution of the lipid precipitated eleven times by methanol was exposed to 0.2 M buffer solutions (acetate, maleate, bicarbonate) at different pH values. The chloroform phase was analyzed for free fatty acid and the aqueous phase for calcium and phosphorus. The pK of the complex was determined from these dissociation data and was taken to be

that point at which 50 per cent of the lipid was dissociated (Fig 1) The shape of the curves is virtually identical for the liberation of fatty acid,

TABLE II  
*Effect of Methanol Precipitation on Chemical Composition  
of Fecal Phosphorus-Containing Lipide*

Ppt No	Total P	Hydroly- sate P	Total Ca	Hydrolysate Ca	Total ash	Total fatty acid*	Specific activity	
							C p m per γ of P	C p m per mg of lipide
	per cent	per cent	per cent	per cent	per cent	per cent		
Original	1 52	1 51	6 70	6 20	15 38	83 49	56 3	855
1	1 61	1 65	6 80	6 40	18 86	78 49	54 8	883
2	2 44	2 46	9 30	8 60	21 40	77 56	52 6	1281
3	2 94	2 78	9 81	9 24	25 65	75 78	48 1	1412
4	3 09	3 15	10 31	9 83	26 33	74 15	50 4	1559
5	3 53	3 48	10 51	10 33	27 23	70 51	44 7	1577
6	3 72	3 88	11 04	11 32	29 08	70 25	45 3	1685
7	4 21	4 17	11 83	11 41	32 35	69 92	46 5	1780

\* Average molecular weight of fatty acid by titration, 289, iodine number, 84 0

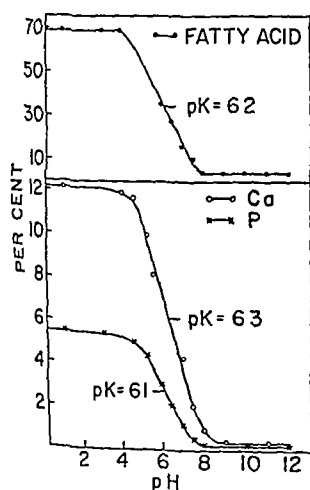


Fig 1

Fig 1 The per cent of P, Ca, and fatty acid liberated from the lipid at different pH levels

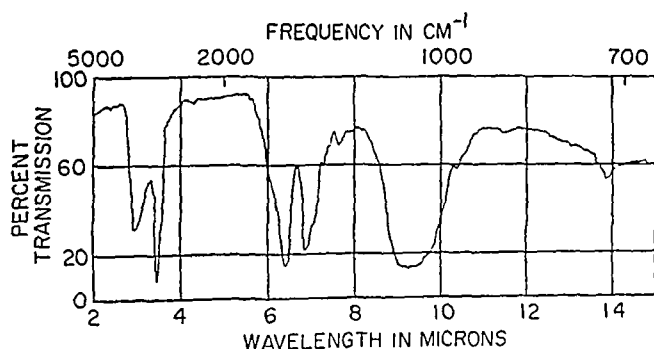


Fig 2

Fig 2 Infrared absorption spectrum of the lipid in the dry state

Ca, and P at different pH levels The pK for the dissociation of the lipid was found to be 6 0 to 6 3, as indicated in Fig 1 No dissociation occurred above pH 7 5 In some experiments the lipid was heated for 16 hours with 10 per cent NaOH and no dissociation occurred

The lipid was subjected to infrared analysis to determine more conclusively the presence and absence of specific bonds and groupings. Its spectrum of the lipid (Fig. 2) was obtained as a KBr pellet by using a Perkin-Elmer recording infrared spectrophotometer with an NaCl prism. The bands in the spectrum indicate the presence of carboxyl ion ( $6.4 \mu$ ), inorganic phosphate ( $9.5 \mu$ ), C—H stretching ( $3.4 \mu$ ), O—H stretching ( $3.0 \mu$ ), and  $(CH_2)_n$  groupings ( $13.8 \mu$ ). The O—H stretching band at approximately  $3.0 \mu$  may be due to either ROH or HOH. Since no alcohol was found in the lipid, the band must be due to water. It is of significance that no bands were present at  $4$  to  $6 \mu$ , indicating the absence of ester, anhydride, aldehyde, and ketone linkages.

In order to obtain more information regarding the formation of the lipid P-containing material,  $P^{32}$  was administered intraperitoneally at

TABLE III  
*Incorporation of  $P^{32}$  into Fecal Phosphorus-Containing Lipide*

Group No *	$P^{32}$ activity	Diet eaten per rat	Per cent total activity recovered in lipid	C p m per $\gamma$ of lipid	C p m per $\gamma$ of lipid after purification
	$\mu c$	gm			
I (4 rats)	100	10	0.7	2.2	113
II (4 " )	100	10	10.1	282	1611

\* Group I,  $P^{32}$  given intraperitoneally, Group II,  $P^{32}$  given in the diet

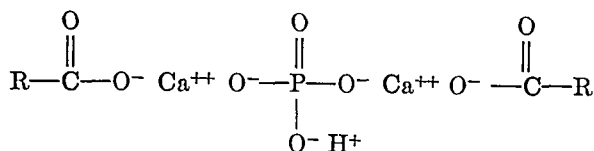
† After purification by methanol precipitation

in the diet (Table III). It is seen that only 0.7 per cent of the  $P^{32}$  activity could be recovered in the lipid when the tracer was given intraperitoneally, whereas 10.1 per cent was recovered when the  $P^{32}$  was administered in the diet. After purification by methanol precipitation, the specific activity was considerably greater in the group which was fed  $P^{32}$  in their diet. These results indicate that the lipid is formed predominantly in the gut from dietary  $PO_4$ , Ca, and fatty acid.

#### DISCUSSION

The present study has confirmed and extended the observations of Ivy and Ivy (1) and Pihl (2). Rats fed an oleic acid diet excreted large amounts of a phosphorus-containing lipid in their feces. From the chemical composition, the dissociation curves, the infrared spectrum, and other data presented, a structure may be proposed for this lipid in which

there are 2 atoms of  $\text{Ca}^{++}$ , 1 mole of  $\text{HPO}_4^-$ , and 2 moles of fatty acid (oleic) as follows



The percentage composition would be calcium 10.8 per cent, phosphorus 4.2 per cent, and fatty acid 76.2 per cent. This is in close agreement with the analytical data. A lipid of this nature has not been reported previously, and its properties of solubility are unusual since it does not contain a covalent phosphate ester bond. The molecular weight of the compound would be 738. Attempts were made to determine the molecular weight of the complex by depression of freezing point in benzene, but values were exceedingly high (3000), suggesting lipid association in this solvent.

The lipid could not be purified to constant composition, but if the material is a salt, then it may be assumed that it was partially ionized in methanol and small amounts of fatty acid were washed out during successive precipitations. Acetone treatment did not reduce the fatty acid content of the lipid as much as methanol, probably because acetone is the less polar of the two solvents.

The  $\text{P}^{32}$  experiments demonstrated that the lipid was formed predominantly in the gut from dietary phosphorus, calcium, and fatty acids. The incorporation of  $\text{P}^{32}$  into the lipid was about fifteen times greater when the isotope was given in the diet than when given intraperitoneally. Thus, there would appear to be no turnover of phosphorus in the lipid and its formation would seem to be strictly exogenous in nature.

It is of interest to note from the previous work (1, 2), and also from experiments recently completed in these laboratories, that oleic acid had the greatest effect on the excretion of this lipid in the feces. Neutral fat and palmitic acid had little effect. The reason for these differences may be related to the presence of the double bond or to the low melting point of oleic acid.

So far, the function and significance of this lipid are unknown. It was observed in the present study that (1) large amounts of unabsorbed fat are excreted in the feces, and (2) that the lipid could account for 85 per cent of the total fat excretion. These findings are of interest, since oleic acid has been shown to be well absorbed. If this lipid has no function in absorption, then it can be assumed that oleic acid, calcium, and phosphorus absorption are impaired because of its formation.



## SUMMARY

1 Rats fed a 25 per cent oleic acid diet excreted large amounts of phosphorus-containing lipide in their feces

2 The lipide resembled phospholipide in that it contained phosphorus and could be precipitated from chloroform solutions by acetone

3 Characterization of the lipide revealed that it was a salt with the following composition: 2 atoms of  $\text{Ca}^{++}$ , 1 mole of  $\text{HPO}_4^-$ , and 2 moles of oleic acid

4 The administration of  $\text{P}^{32}$  indicated that the lipide was formed in the gut from dietary phosphorus, calcium, and oleic acid. There was no phosphorus turnover in the compound

5 The formation of this lipide greatly impaired the absorption of oleic acid

The infrared analysis and interpretation were made through the courtesy of W. B. Wartman, Jr., Research Laboratory, The American Tobacco Company, Inc., Richmond, Virginia

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# SPECIFIC REACTIONS OF DINITROFLUOROBENZENE WITH ACTIVE GROUPS OF CHYMOTRYPSIN\*

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The sulfhydryl, disulfide (1), phenolic (2), imidazole (3), and amino groups (4) of proteins exhibit varying degrees of reactivity in the native protein. Šorm and Rychlík (5) have shown that the protease and milk-clotting activities of chymotrypsin (ChTr) drop with increasing extent of substitution by 2,4-dinitrofluorobenzene (DNFB, or Sanger's reagent). The esterase activity of ChTr containing two dinitrophenyl (DNP) groups per mole of protein was higher than the control, but the activity decreased on further treatment with DNFB. Massey and Hartley (6) reported that the conditions normally employed in the reaction of proteins with DNFB (7) produce a rapid inactivation of ChTr. Under milder conditions of reaction, only 1 of the 2 histidine residues reacted with DNFB.

The present work was carried out in an attempt to determine whether the reaction of any particular amino acid residue in ChTr is associated with the loss of enzymatic activity. The reaction of ChTr with DNFB has been studied under various conditions of pH, temperature, and molar ratio of reactants. The reaction was followed by correlating loss of enzymatic activity with the amount and distribution of protein-bound DNP residues.

## EXPERIMENTAL

*ChTr*—All the experiments were carried out with a salt-free preparation of ChTr (Mann). The protein concentration was determined by the method of Schwert and Kaufman (8), an average molecular weight of 22,500 was used for ChTr in calculation of molar concentrations of ChTr and its chemically modified forms. Esterase activity was determined by the method of Schwert *et al.* (9) with *N*-acetyltyrosine ethyl ester as the substrate at pH 7.40 (tris(hydroxymethyl)aminomethane (Tris) buffer). The ChTr had an esterase activity of 0.34 m eq. per minute per mg. of protein.

*P<sup>32</sup>-Labeled Diisopropylphosphorofluoridate (DFP<sup>32</sup>)*—This was obtained commercially with an initial specific activity of about 10 mc. per mmole.

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and a purity of 65 per cent. The determination of purity was based on the extent of reaction with a large excess of ChT<sub>i</sub>.

*Dinitrophenyl Chymotrypsin (DNPChTr)*— $3.90 \times 10^{-4}$  M ChTr was treated with a 1 to 27 molar ratio of DNFB (prepared as a dilute solution in methanol) to ChTr in the presence of 0.1 to 0.2 M buffer, 1.0 M KCl, 15 per cent methanol, and at 25.0° for the desired time. The amount of DNFB which had reacted with ChT<sub>i</sub> was determined by measuring the amount of unchanged DNFB. The DNPChTr was precipitated with 5 per cent trichloroacetic acid (TCA) and washed free from DNFB either with acetone or with 1 per cent TCA. The extensively dinitrophenylated ChT<sub>i</sub> is appreciably soluble in acetone, and 1 per cent TCA was used for washing this material. The DNFB was hydrolyzed with 0.2 N NaOH in a water bath for 20 minutes and the color produced by the resultant dinitrophenol measured at 357 mμ with the Beckman DU spectrophotometer.

*P<sup>32</sup>-Labeled Diisopropylphosphoryl (DP)-DNPChTr*— $3.90 \times 10^{-4}$  M ChTr was partially inactivated by reaction with DNFB and then treated, at a concentration of  $2.60 \times 10^{-4}$  M, with  $3.18 \times 10^{-4}$  M DFP<sup>32</sup> for 30 minutes at room temperature and a pH of 7.40. The DP<sup>32</sup>-DNPChTr was precipitated with 5 per cent TCA, and the precipitate was thoroughly washed, rapidly redissolved in 0.1 N NaOH at room temperature (10 seconds), and rapidly reprecipitated with TCA. The precipitate was washed, redissolved in 0.1 N NaOH, and the radioactivity measured on an aliquot of the sample. DNPChTr, but not ChTr itself, has a tendency to occlude or adsorb unchanged DFP<sup>32</sup> and its hydrolysis products upon precipitation with 5 per cent TCA. Conclusions drawn from the radioactivity remaining in the supernatant solution would therefore be erroneous. For example, analysis of the TCA filtrate of a reaction mixture in which ChTr had been completely inactivated by reaction with DNFB, and then treated with a 1.22 molar ratio of DFP<sup>32</sup> to DNPChTr (the actual ratio added was 1.88 of the 65 per cent pure DFP<sup>32</sup>), showed that 1 to 1.5 moles of DFP<sup>32</sup> per mole of ChT<sub>i</sub> had disappeared from the supernatant fluid. However, all of this P<sup>32</sup> could be removed from the precipitate by dialysis against 0.5 M, pH 7.40, phosphate buffer. The adsorbed P<sup>32</sup> can also be removed from samples of partially inactivated DNPChT<sub>i</sub> by rapidly dissolving the precipitate in 0.1 N NaOH at room temperature (approximately 10 seconds) and at once precipitating the protein with TCA. Control experiments have shown that DPChT<sub>i</sub> is stable to this treatment. Unfortunately, completely inactivated DNPChTr is not readily soluble in 0.1 N NaOH at room temperature.

*Hydrolysis of DNPChTr*—The DNPChTr was precipitated with sufficient 50 per cent TCA to make a final concentration of 5 per cent TCA,

washed free from DNFB and dinitrophenol as described above, and dried in a vacuum desiccator over  $\text{CaCl}_2$ . The material was hydrolyzed by incubation in a boiling water bath for 16 hours with 10 times its weight of 6 N HCl. The excess HCl was removed by repeated concentration in a vacuum desiccator over NaOH and the acidic solution extracted three times with ether.

*Dinitrophenyl Chymotrypsinogen (DNPChTrogen)*—This material was prepared and hydrolyzed in the same manner as described for DNPChTr.

*Separation and Determination of Histidine*—The histidine was separated from the other amino acids by the method of Winters and Kunin (10). The acid was removed by passing the hydrolysate through a column of Amberlite IR-4B in the hydroxyl form. Lysine and arginine were then adsorbed on a column of Amberlite IRC-50 buffered at pH 7.0, and histidine on a column of Amberlite IRC-50 buffered at pH 4.6 with acetate buffer. The histidine was eluted with 1 N HCl and determined quantitatively by the *p*-nitrobenzoyl chloride method of Cowgill (11).

### Results

The reaction of ChTr with DNFB was carried out in 1 M KCl for the following reason. It was noted that, when a sample of partially inactivated DNPChTr was allowed to stand in 1 M KCl-0.3 per cent gelatin solution (this solution was used to dilute the enzyme to the proper level for activity determinations), the activity gradually increased (Fig. 1). However, when the reaction with DNFB was carried out in the presence of 1 M KCl, the activity of the diluted DNPChTr remained about constant over a 24 hour period (Fig. 2) and the presence or absence of KCl had little or no effect on the rate of reaction with DNFB. The explanation for this increase in activity of partially inactivated DNPChTr is not available at the present time. Controls carried out under identical conditions did not show this phenomenon.

The reaction of ChTr with DNFB is rather slow at 25.0° and pH values close to neutrality. Significantly higher temperatures could not be used since, in control experiments carried out under the conditions used for reaction with DNFB, ChTr was rapidly inactivated at 35.0°. Thus, at the concentration levels of ChTr and DNFB ( $3.90 \times 10^{-4}$  M and  $1.05 \times 10^{-2}$  M, respectively) used in Fig. 3, it requires approximately 9 hours to produce a 50 per cent loss in activity. Inactivation of ChTr begins only after the addition of 2 moles of DNFB per mole of ChTr, and no increase in original activity as reported by Šorm and Rychlík (5) was found. At this pH, the specificity of DNFB towards the "active center" of ChTr appears to be very small. From Mills' work with insulin (12), it was to be expected that changes in pH would have a differentiating effect on the

reactivity of individual residues of ChTr towards DNFB. For these reasons it was decided to study the reaction of ChTr with DNFB over the pH range 6 to 11.

Since both phosphate and borate buffers were needed to cover the entire range, the effect of buffer ions on reactivity and stability of ChTr under the conditions of the reaction had to be studied<sup>1</sup>. It may be seen from

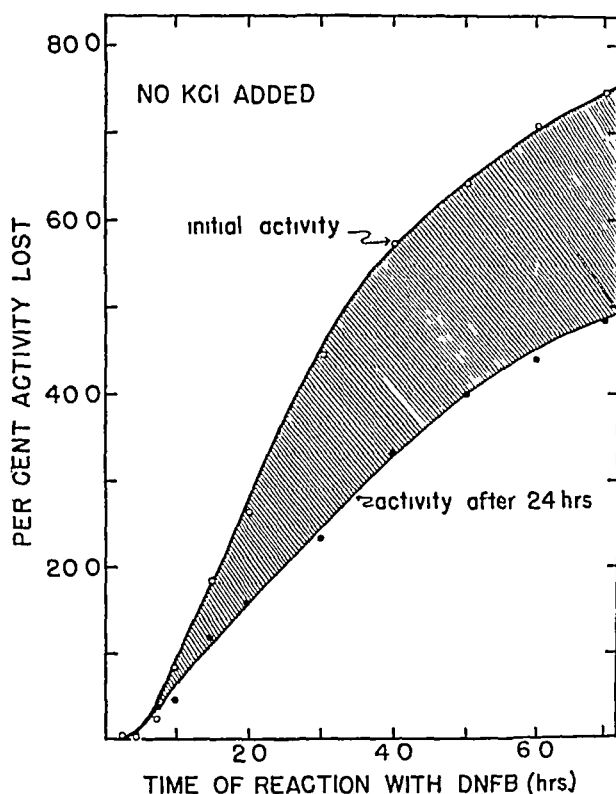


FIG. 1. Gain of activity of partially inactivated DNPChTr on standing in 1 M KCl-0.3 per cent gelatin solution.  $3.90 \times 10^{-4}$  M ChTr was treated with  $6.75 \times 10^{-3}$  M DNFB in 0.2 M, pH 7.50, Tris buffer, 5 per cent methanol and no KCl at 25.0°.

Table I that ChTr, at a concentration of  $3.90 \times 10^{-4}$  M, reacted more rapidly and was more resistant to denaturation in the borate and phosphate buffers than in phosphate buffer. Because of this effect of phosphate ions on the stability and reactivity of ChTr, the buffer systems from pH 7.21 through 9.15 were prepared from equimolar mixtures of phosphate and borate buffers.

Table II shows the stability of  $3.90 \times 10^{-4}$  M ChTr over the pH range 6.20 to 11.07. The increased rate of inactivation at pH 8.77 is presumably

<sup>1</sup> The use of Tris buffer was abandoned after the initial experiments because the buffer itself slowly reacts with DNFB.

due to the fact that this pH is optimal for autolysis ChT<sub>1</sub>, under the conditions used here, is rather stable at pH 10 to 11 and short time reactions with DNFB may be carried out at this pH with little or no loss of activity from denaturation

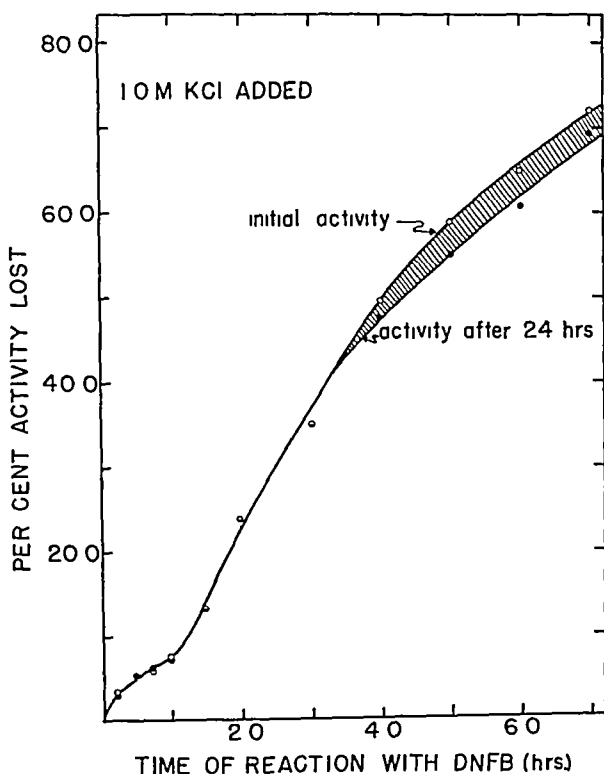


FIG. 2. Gain of activity of partially inactivated DNPChTr on standing in 1 M KCl 0.3 per cent gelatin solution.  $3.90 \times 10^{-4}$  M ChTr was treated with  $1.05 \times 10^{-2}$  M DNFB in 0.2 M, pH 7.50, Tris buffer, 5 per cent methanol and 1.0 M KCl at 25.0°

The reaction rate of DNFB with ChTr was found to be a constant at a given pH when calculated according to the equation

$$K = \frac{1}{t} \frac{x}{a(a-x)}$$

where  $K$  is the specific reaction rate constant,  $t$  is the time of reaction with DNFB,  $x$  is the number of groups which reacted with DNFB at time  $t$ , and  $a$  is the number of DNFB-reactive groups on ChT<sub>1</sub>. This is the form of the second order rate equation in which the concentrations of the two reactants are similar. There are twenty-two groups on ChT<sub>1</sub> capable of reacting with DNFB (7, 13), and 27 moles of DNFB per mole of ChTr were initially available for reaction. As shown by Fig. 4, the pH of the reaction mixture has a marked influence on the rate of reaction

between DNFB and ChTr Under the conditions used, the reaction at a pH higher than 9.15 is too rapid for convenient measurement.

Not only was the reaction of ChTr with DNFB more rapid at a higher pH, as one might expect, but it was found that the reaction becomes more specific. As the pH increases, the number of moles of DNFB required to be bound per mole of ChTr to produce 50 per cent inactivation decreases, with a minimum at pH 10.66 (Fig. 5). It should be noted that the points

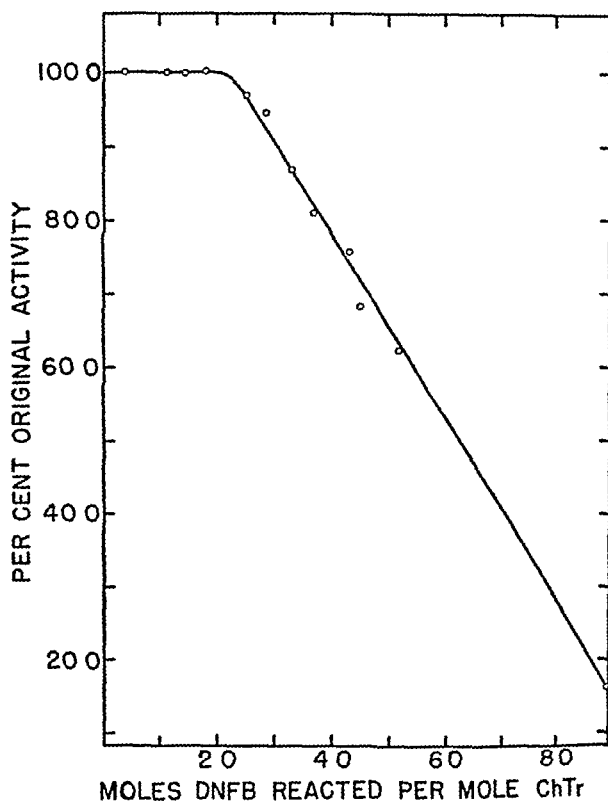


FIG. 3 Rate of inactivation of  $3.90 \times 10^{-4}$  M ChTr with  $1.05 \times 10^{-2}$  M DNFB in 0.2 M, pH 7.24, phosphate buffer, 10 per cent methanol and 1.0 M KCl at 25.0°

above pH 10 were obtained by means of a 3-fold rather than the usual 27-fold molar ratio of DNFB to ChTr because of the excessive speed of reaction in this pH range when the higher ratio of DNFB to ChTr is used. The observed  $I_{50}$  would be larger if the 27-fold molar ratio of DNFB to ChTr were used (note the use of 7.2 molar ratio of DNFB to ChTr in Fig. 5). But the data clearly indicate that the point of maximal specificity is near pH 10.66. At this pH, the addition of 1.06 moles of DNFB per mole of ChTr results in over 50 per cent inactivation with binding of less than 1 mole of DNFB per mole of ChTr.<sup>2</sup>

<sup>2</sup> At pH 10.66, more DNFB may be found bound to ChTr during the 1st few minutes than after the reaction has proceeded for 2 to 4 hours, even though the activity

Diisopropylphosphorofluoridate (DFP) reacts with ChTr on an equimolar basis (15). Is the inactivation of ChTr by DFP and DNFB caused by

TABLE I  
*Comparison of Effect of Phosphate and Borate Buffers  
on Reaction of DNFB with ChTr*

Time	Controls		With DNFB					
	Per cent activity lost		Phosphate buffer 0.2 M, pH 7.97		Borate buffer 0.2 M, pH 7.98		0.1 M phosphate 0.05 M borate buffer pH 7.95	
	Phosphate buffer 0.2 M pH 7.97	Borate buffer 0.2 M pH 7.98	Per cent activity lost	Moles DNP bound per mole ChTr	Per cent activity lost	Moles DNP bound per mole ChTr	Per cent activity lost	Moles DNP bound per mole ChTr
hrs								
0.50			6.4	3.05	15.5	4.34	15.0	3.66
1.0	4.4	0.0	24.0	4.88	41.2	6.60	39.0	5.24
2.0			55.1	7.92	78.0	9.30	71.7	8.65
3.0	9.2	2.0						
4.0			85.3	11.18	92.7	13.29	92.2	13.19
5.0	16.0	4.2						
24.0	36.8	12.9						

$3.90 \times 10^{-4}$  M ChTr was treated with  $1.05 \times 10^{-2}$  M DNFB in 15 per cent methanol, 1.0 M KCl, and the appropriate buffer at 25.0°

TABLE II  
*Loss of Activity of Chymotrypsin at Various pH Values*

Time	Per cent activity lost										
	pH 6.20	pH 6.97	pH 7.21	pH 7.51	pH 7.95	pH 8.25	pH 8.77	pH 9.15	pH 10.03	pH 10.66	pH 11.07
hrs											
2.0	0.0	0.0	0.5	1.6	1.4	1.4	3.0	0.0	0.3	1.6	2.6
6.0	0.0	0.0	0.3	3.3	3.9	3.6	8.5	3.8	6.5	13.0	15.5
24.0	5.5	8.7	6.5	8.4	8.5	9.0	12.4	11.7	17.3	29.0	31.5

$3.90 \times 10^{-4}$  M ChTr in 1.0 M KCl, 15 per cent methanol, and the proper buffer. Buffers used, pH 6.20, 6.97, 0.2 M phosphate pH 7.21 to 9.15, 0.1 M borate 0.1 M phosphate pH 10.03, 0.2 M borate pH 10.66, 11.07, 0.1 M carbonate

reactions with the same or a different group on the ChTr molecule? To answer this question, ChTr was first partially inactivated by DNFB and of the DNPChTr continues to decrease because of additional reaction with the imidazole group (Tables III, IV, V). This is thought to be due to the instability of ε-DNP lysine in alkaline solution (14).



then treated with  $P^{32}$ -labeled DFP. The extent of inactivation of ChTr with DNFB ranged from a few per cent to complete inactivation. These reactions were carried out at both pH 7.51 and 10.66 to make sure that the mechanism of the reaction of ChTr with DNFB which produces enzyme inactivation was the same under varying conditions of specificity. The reaction with  $P^{32}$ -labeled DFP was carried out in Tris buffer, pH 7.40,

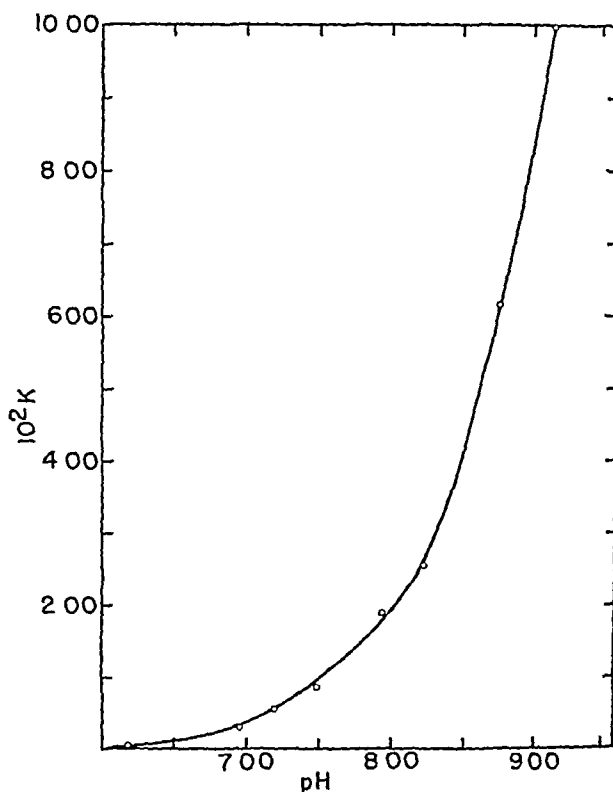


FIG. 4. Effect of pH on the reaction of ChTr with DNFB.  $3.90 \times 10^{-4}$  M ChTr was treated with  $1.05 \times 10^{-2}$  M DNFB in 0.2 M buffer, 15 per cent methanol, 1.0 M KCl and at  $25.0^\circ$ . The buffers were pH 6.20 and 6.97, 0.2 M phosphate buffers, pH 7.21 through 9.15, 0.1 M borate-0.1 M phosphate buffers.

and a molar ratio of  $DFP^{32}$  to ChTr of 1.22. Tables III and IV show that the amount of  $DFP^{32}$  capable of reaction with the partially inactivated DNPChTr is the same as the amount of enzymatic activity remaining after reaction with DNFB. These results lead to the conclusion that DFP and DNFB react with one and the same grouping on the ChTr molecule.

The amount of free histidine remaining in ChTr after partial inactivation with DNFB was determined. Close agreement was found between the fraction of 1 mole of histidine which reacted with DNFB and the loss of esterase activity (Table V). There are 2 histidine residues per mole of

ChTr (13), but it appears that only one of these is capable of reaction with DNFB under the experimental conditions used

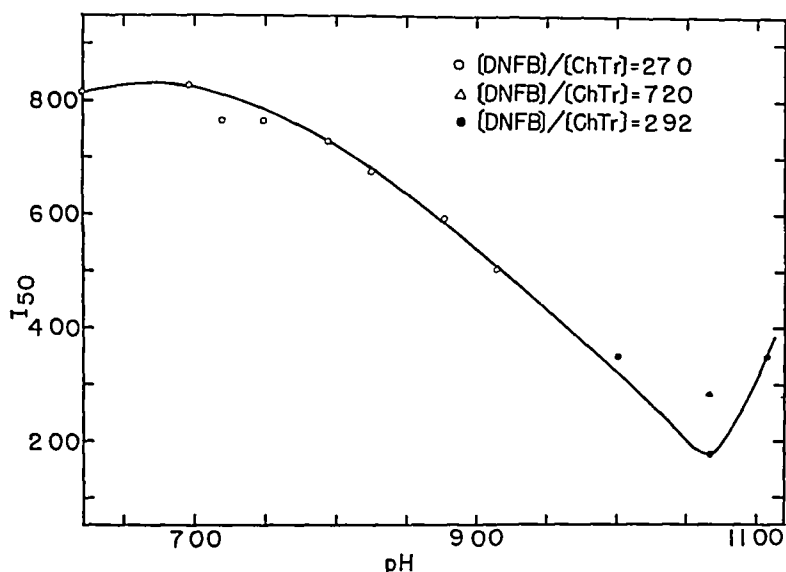


Fig 5 Number of moles of DNFB per mole of ChTr required to produce 50 per cent inactivation ( $I_{50}$ ) as a function of pH.  $3.90 \times 10^{-4}$  M ChTr was treated with the concentration of DNFB given. The buffers were pH 6.20 and 6.97, 0.2 M phosphate buffers, pH 7.21 through 9.15, 0.1 M borate-0.1 M phosphate buffers, pH 10.03, 0.2 M borate buffer, and pH 10.66 and 11.07, 0.1 M carbonate buffers.

TABLE III  
*Reaction of DNPChTr with DFP<sup>32</sup>*

Time of reaction with DNFB	Fraction of ChTr active after reaction with DNFB	Moles DFP <sup>32</sup> bound per mole ChTr	Moles DNP bound per mole ChTr
<i>hrs</i>			
0.0	1.000	0.996	
1.0	0.934	0.926	2.40
2.0	0.755	0.779	4.60
3.0	0.585	0.547	6.63
4.0	0.455	0.459	8.20
5.0	0.322	0.382	9.79
6.0	0.213	0.269	11.07

$3.90 \times 10^{-4}$  M ChTr was partially inactivated by reaction with  $1.05 \times 10^{-2}$  M DNFB at pH 7.51 (0.1 M borate-0.1 M phosphate buffer), in 15 per cent methanol and 1.0 M KCl at 25.0° and then treated for 30 minutes at 25.0° and pH 7.40 ([ChTr] =  $2.60 \times 10^{-4}$  M) with  $3.18 \times 10^{-4}$  M DFP<sup>32</sup>.

When chymotrypsinogen (ChTrogen) is incubated with a 3-fold molar ratio of DNFB to ChTrogen under the same conditions as ChTr, 2.23 moles of DNFB react per mole of ChTrogen, but no DNFB is found bound

to histidine (Table VI) DNPChTrogen is activated by trypsin at the same rate as is ChTrogen, without loss of DNP residues, and the specific

TABLE IV  
Reaction of DNPChTr with DFP<sup>32</sup>

(DNFB)/(ChTr) = 1.06				(DNFB)/(ChTr) = 2.93			
Time of reaction with DNFB	Fraction ChTr active after reaction with DNFB	Mole DP <sup>32</sup> bound per mole ChTr	Moles DNP bound per mole ChTr	Time of reaction with DNFB	Fraction ChTr active after reaction with DNFB	Mole DP <sup>32</sup> bound per mole ChTr	Moles DNP bound per mole ChTr
<i>min</i>				<i>min</i>			
15	0.850	0.843	0.79	45	0.534	0.554	1.72
				<i>hrs</i>			
30	0.774	0.745	0.71	2	0.360	0.392	1.58
60	0.686	0.680	0.70	4	0.266	0.281	1.49
10*	0.056	0.062	14.20				

$3.90 \times 10^{-4}$  M ChTr was partially inactivated by reaction with DNFB at pH 10.66 (0.1 M carbonate buffer), in 15 per cent methanol and 1.0 M KCl at 25.0°, then treated for 30 minutes at 25.0° and pH 7.40 ((ChTr) =  $2.60 \times 10^{-4}$  M) with  $3.18 \times 10^{-4}$  M DFP<sup>32</sup>.

\* In this run (DNFB/ChTr) was 27.0, the final mixture, after reaction with DFP<sup>32</sup>, was dialyzed 5 days against 0.5 M, pH 7.50, phosphate buffer.

TABLE V  
Free Histidine Content of ChTr Partially Inactivated with DNFB

Time of treatment with DNFB	Moles DNP bound per mole ChTr	Moles free histidine per mole ChTr	Fraction of 1 mole free histidine disappeared	Fraction of activity lost
<i>min</i>				
0.0		1.995		
15.0	0.70	1.839	0.156	0.145
45.0	1.75	1.546	0.449	0.440
<i>hrs</i>				
1.0	0.61	1.635	0.360	0.354
2.0	1.57	1.349	0.646	0.613
4.0	1.53	1.229	0.766	0.744

$3.90 \times 10^{-4}$  M ChTr was treated with  $3.50 \times 10^{-4}$  M (for 15 minutes and for 1 hour) and  $1.05 \times 10^{-3}$  M (for 45 minutes and for 2 and 4 hours) DNFB at pH 10.66 (0.1 M carbonate buffer) in 1.0 M KCl, 15 per cent methanol, and at 25.0°.

esterase activities of the resultant DNPChTr and ChTr are the same. In a separate experiment, ChTrogen was treated with a larger amount of DNFB (DNFB/ChTrogen = 9) for 45 minutes under the same conditions as described in Table VI. Approximately 80 per cent of the protein became insoluble during this reaction, which led to the uptake of

775 DNP residues per mole of ChTrogen. However, the *total* protein (soluble and insoluble) still contained 20 moles of free histidine per mole of protein as determined after hydrolysis. The soluble portion, containing 675 DNP residues per mole of protein, exhibited the same specific activity after trypsin activation as a control which had not been treated with DNFB. This would indicate that dinitrophenylation of ChT<sub>1</sub> at sites other than the reactive histidine residue has no effect on its activity.

The bond formed by the reaction of DFP with ChT<sub>1</sub> is not stable to the acid hydrolysis used here, and all of the histidine is found in the unbound form when it is separated from an acid hydrolysate of DPChT<sub>1</sub>. When DPChT<sub>1</sub> is allowed to react immediately with a 3-fold molar ratio

TABLE VI  
*Reaction of ChTrogen with DNFB*

Time of reaction with DNFB	Moles DNP bound per mole ChTrogen	Moles free histidine per mole Ch Trogen after reaction with DNFB	Rate of conversion to DNPChTr and ChTr, per cent							
			Compound	2 min	4 min	8 min	16 min	30 min	40 min	60 min *
2 0 hrs	2 23	1 997	ChTrogen	24 0	34 7	53 3	73 2	87 9	95 5	100 0
			DNPChTrogen	24 3	36 1	52 3	74 4	91 6	100 0	100 0

$3.90 \times 10^{-4}$  M ChTrogen was treated with  $1.05 \times 10^{-3}$  M DNFB for 4 hours at pH 10.66 (0.1 M carbonate buffer), in 1.0 M KCl, 15 per cent methanol, and at 25.0°. DNP-ChTrogen and ChTrogen ( $1.67 \times 10^{-4}$  M) were converted to DNPChTr and ChTr by  $1.67 \times 10^{-5}$  M trypsin at 21° and pH 7.6. The solution contained 0.43 M KCl, 6.40 per cent methanol, 0.043 M carbonate, 0.29 M phosphate, and 0.43 M Tris buffer.

\* The activity after 60 minutes (unchanged after 19 hours) was taken as 100 per cent.

of DNFB to DPChTr for 45 minutes at pH 10.66, and then hydrolyzed, all of the histidine is separated as free histidine. The same result is obtained with DPChTr which has aged for 6 days before being treated with DNFB.

#### DISCUSSION

It is not clear why the reactivity of the imidazole N (pK approximately 6.1) towards DNFB should be so greatly increased at high pH values. It must be associated with the nature of the "active center," as the second imidazole group does not behave in this fashion. Evidently, the DNFB is acting in the role of a substrate for ChTr but, once bound, like the organophosphorus compounds, it cannot be released from the "active center." Unlike the organophosphorus compounds, however, the initially formed DNPChTr is stable and the DNP group is not transferred to a second amino acid residue. Although it has been found that, when ChTr is

completely inactivated with DFP or Sarin,<sup>3</sup> the phosphorus is bound to the hydroxyl group of a serine moiety (16, 17), there is evidence for a sequential reaction. On prolonged incubation of an organophosphorus inactivated enzyme, the enzyme becomes more resistant to reactivation by hydroxamic acids (18-20). This phenomenon is dependent on temperature and has been interpreted as indicating a gradual transfer of the alkylphosphoryl group from an unstable to a more stable linkage (20). The finding that DNFB does not react with the histidine residue of aged DPChTr does not disprove the theory of a sequential reaction for at least two reasons. First, the hydroxyl group of serine where the DP group is eventually found may be so close spatially to the imidazole group of histidine as to prevent a reaction between DNFB and histidine, secondly, the imidazole group of the "reactive histidine" may no longer react as readily with DNFB after the protein has lost its enzymatic properties.

The possibility of histidine being involved in the "active center" of ChTr and in the first stage of the postulated sequential reaction is supported by various lines of evidence. Weil *et al* (21) found that the gradual loss of ChTr activity produced by photooxidation in the presence of methylene blue was associated with the loss of histidine and tryptophan. After complete inactivation, 1 mole of histidine and 3 moles of tryptophan were lost, with little or no loss of the other amino acids measured. This finding has been confirmed by Jandorf *et al* (20), who also reported that the rate of oxygen uptake and histidine and tryptophan loss is lower for DPChTr than for ChTr, and that photooxidation results in no loss of protein-bound DFP-derived phosphorus.

Weil and Seibles (22) also found a correlation between the loss of activity and the loss of histidine on the photooxidation of ribonuclease (ribonuclease does not contain tryptophan). However, Wood and Balls (23) found that ChTr was inactivated by treatment with peroxidase, which caused the loss of 1 mole of tryptophan but had no effect on histidine or tyrosine. Because the inactive ChTr would still react with diphenylphosphorochloridate to the same extent as untreated ChTr, they concluded that the tryptophan residue lost acted "as an auxiliary rather than a necessary group in the action of the enzyme" (23). Gross and Egan (24) reported that the complete reduction of the disulfide groups of ChTr with *p* chloromercuribenzoate resulted in complete loss of enzymatic activity. However, it also resulted in extensive destruction of the ChTr molecule. Histidine is the only naturally occurring amino acid found to catalyze the hydrolysis of DFP at the P—F bond (25). Massey and Hartley (6), by the use of DNFB, have shown that 1 of the 2 histidine residues of ChTr is associated with the activity.

<sup>3</sup> Isopropyl methylphosphonofluoridate

The amount of DNFB bound to the imidazole group of histidine accounts for only a part of the total DNFB bound under the conditions used. For example, in the treatment of DNFB with ChT<sub>1</sub> for 1 hour (Table V), 0.61 mole of DNFB per mole of ChT<sub>1</sub> reacts but only 0.360 mole is bound to histidine. Optical density measurements of the precipitate, dissolved in 0.2 N NaOH, read at 357 m $\mu$  and with  $\epsilon$ -DNP lysine as a standard, showed 0.25 mole of DNFB bound per mole of ChT<sub>1</sub>. The calculations from data by Levy and Li (26) result in a value of 0.22 mole of DNFB per mole of ChTr for the combined reaction with alanine, isoleucine, and lysine. The difference of 0.03 mole of DNFB, if significant, might be accounted for by reaction with the hydroxyl group of tyrosine, as (O)-DNP tyrosine is colorless, in analogy with the demonstrated, albeit slow, reaction of phenolic hydroxyl groups in proteins and model compounds with organophosphorus compounds (20, 27, 28).

The 1 histidine residue that reacts with DNFB in ChT<sub>1</sub> does not react in ChTrogen. One must conclude, therefore, that the imidazole group of this histidine is "covered up" in some manner and released when ChT<sub>1</sub>-ogen is converted to ChT<sub>1</sub>.

#### SUMMARY

Chymotrypsin is inactivated by reaction with 2,4-dinitrofluorobenzene. This reaction becomes more rapid and more specific at higher pH values and reaches a point of maximal specificity at pH 10.66. On treatment of chymotrypsin with 2,4-dinitrofluorobenzene under mild conditions, the decrease in enzymatic activity parallels the fraction of 1 mole of histidine bound by 2,4-dinitrofluorobenzene, and also parallels the same fractional loss in the diisopropylphosphorofluoridate-binding ability of the enzyme. It appears, therefore, that diisopropylphosphorofluoridate and 2,4-dinitrofluorobenzene cause inactivation of chymotrypsin by reaction with the same group and that 1 of the 2 histidine residues in chymotrypsin is associated with this "active center." Dimethylphenylation of chymotrypsinogen does not involve histidine, the resulting protein is activated by trypsin at the same rate, and to the same specific activity, as untreated chymotrypsinogen.

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# STUDIES ON URIC ACID AND RELATED COMPOUNDS

## III OBSERVATIONS ON THE SPECIFICITY OF MAMMALIAN XANTHINE OXIDASES

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Xanthine oxidase (XO) has been thoroughly studied by many investigators and the range of possible substrates of this enzyme is well known (1, 2). In the early experiments, enzymatic activity was measured by decolorization of a suitable dyestuff such as methylene blue or by oxygen consumption. Therefore, the exact pathway of oxidation remained unknown for many substrates. The spectrophotometric method of Kalckar (3, 4), which is now being widely applied to hypoxanthine and xanthine, is much more specific. This method has been extended in the present and the following papers to identify the oxidation products of various purine derivatives unequivocally. In this way, the substrate specificity and other characteristic properties of xanthine oxidase have been determined. Our results enable us to draw certain conclusions about the mode of attachment of the substrate to the active center of the enzyme and about the mechanism of the dehydrogenation catalyzed by it. We have also found that among all methylated uric acids, which are formed after administration of methylated xanthines to animals, only the 1-methyl derivative can be produced by direct action of XO. Thus, all other substituted uric acids must result from a different biochemical pathway. This problem will be dealt with in Paper IV.

### EXPERIMENTAL

*Materials*—Purine was supplied by Dr. Aaron Bendich of the Sloan-Kettering Institute for Cancer Research. The 2- and 8-hydroxypurines, and likewise the 2,8- and 6,8-dioxypurines, were obtained through the courtesy of Professor Adrian Albert of the Australian National University, Canberra. Both 1- and 7-methylhypoxanthines were a gift from Dr. Gertrude B. Elion of the Wellcome Research Laboratories, Tuckahoe, New York. 3-Methylxanthine was given to us by Dr. V. Papesch of G. D. Searle and Company, Chicago, and the 7-methyl and 1,7-dimethyl derivatives by Dr. J. J. Fox of the Sloan-Kettering Institute for Cancer

\* Part of a thesis for the degree of Doctor of Philosophy, submitted to the Faculty of Science, The Hebrew University, Jerusalem, 1957.



Research 1-Methylxanthine was synthesized by us as reported previously (5)

*Enzymes*—Milk xanthine oxidase was a gift of Professor F Bergel and Dr R C Bray of the Chester Beatty Institute of Cancer Research, London (6) At pH 8.3, the preparation, when diluted 1/400, produced 1.0  $\gamma$  of uric acid per ml per minute when  $10^{-4}$  M xanthine served as substrate Human liver xanthine oxidase was purified according to the method of Kielley (7) Under the same conditions as before, a 1/20 dilution of this enzyme produced 0.2  $\gamma$  of uric acid per ml per minute

### *Methods of Analysis*

Uric acid production was measured at 300  $m\mu$ , although the extinction is only about 85 per cent of that at 290  $m\mu$ , where readings are usually

TABLE I  
*Optical Density of Uric Acid ( $10^{-4}$  M) As Function of pH*

pH	Optical density at	
	290 $m\mu$	300 $m\mu$
5.5	1.15	0.75
6.4	1.165	0.89
7.1	1.18	0.99
8.0	1.20	1.0
9.0	1.20	1.0
9.9	1.20	1.0

taken Xanthine at pH 8.0, in  $10^{-4}$  M concentration, shows an optical density of 0.1 at 300  $m\mu$  as against 0.42 at 290  $m\mu$  An equimolar solution of uric acid possesses an extinction of 1.0 at 300  $m\mu$  and of 1.20 at 290  $m\mu$  Therefore, enzymatic conversion of xanthine into uric acid at 300  $m\mu$  requires a correction factor of 1.1 only and is thus much more reliable

In Table I we show the variation of optical density with pH These figures have been used to calculate the amount of uric acid formed at pH 5.5 and 6.4 At the other pH values the optical density is constant

2,8-Dioxypurine was determined at 320  $m\mu$  As is evident from Fig. 1, it is possible to identify this compound unequivocally in the presence of 8-oxypurine and uric acid, which do not absorb at such a high wave length It is more difficult to analyze a mixture of 2-oxy- and 2,8-dioxypurines because of the similarity of their absorption curves in the region between 280 and 340  $m\mu$  (Fig. 1) In the experiments on the oxidation of 2-oxypurine readings were taken at 317  $m\mu$ , the isosbestic point of this substrate with 2,8-dioxypurine, where formation of uric acid lowers the optical

density considerably, and at 303  $m\mu$ , where 2,8-dioxyurine and uric acid possess an isosbestic point, hence any increase in optical density measures their sum. Thus the amount of 2,8-dioxyurine and of uric acid, or of both, formed from 2-oxyurine can be determined quantitatively.

Oxidation of 1-methylxanthine was measured as uric acid, since the latter cannot be distinguished spectrophotometrically from its 1-methyl derivative at any pH. However, the nature of the oxidation product

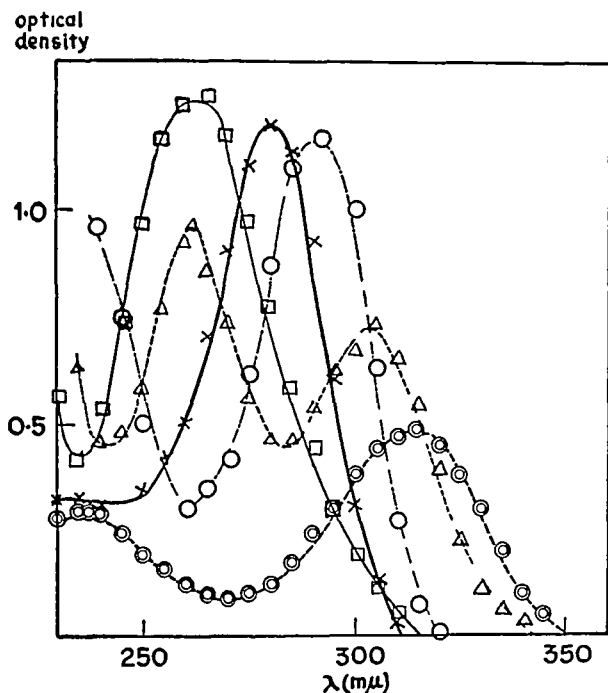


FIG 1 Absorption spectra of various purine derivatives at pH 8.0. Concentration used,  $10^{-4}$  M.  $\odot$ , 2-oxyurine,  $\times$ , 8-oxyurine,  $\triangle$ , 2,8-dioxyurine,  $\square$ , 6,8-dioxyurine,  $\circ$ , uric acid. The isosbestic points used in this paper are 317  $m\mu$ , 2-oxyurine and 2,8-dioxyurine, 303  $m\mu$ , 2,8-dioxyurine and uric acid, 295  $m\mu$ , 8-oxyurine and 2,8-dioxyurine.

could be established unequivocally by paper chromatography (5) (see under "Results").

Purine oxidation was followed up by measuring through the whole spectral range between 270 and 320  $m\mu$ .

*Procedure*—An appropriate dilution of the enzyme, 0.1 M phosphate buffer of pH 8.0 (*i.e.* near the pH optimum), and the substrate,  $10^{-4}$  M, were mixed at room temperature (21°) at zero time in a quartz cell, and the optical density of the mixture was read in a Beckman ultraviolet spectrophotometer, connected to a photomultiplier, at suitable intervals. For substrates with very low activity, the observation time had to be

extended over many hours. In order to avoid bacterial infection, a drop of chloroform was added after we had found that this substance does not affect enzymatic activity.

For the determination of pH-activity curves the following buffers were used: pH 5.5 to 6.6, 0.1 M acetate; pH 7.7 to 8.3, 0.1 M phosphate; pH 8.6 to 10.0, 0.025 M pyrophosphate.

In control experiments it was shown that the above concentration of acetate does not produce measurable inhibition.

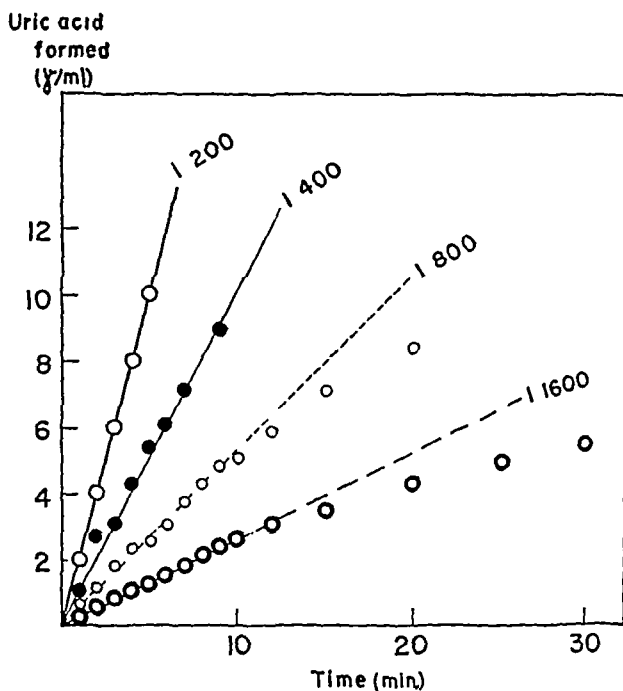


FIG. 2. Rate of oxidation of xanthine as function of time for various dilutions of xanthine oxidase. Substrate concentration,  $10^{-4}$  M. The curves of this graph demonstrate that the rate is linear only for the first 10 minutes.

The formation of uric acid at various enzyme dilutions is shown in Fig. 2. It is apparent that after 10 minutes the rate decreases, probably because of enzyme inactivation by  $H_2O_2$  (8). Therefore, all rates in this paper represent the values obtained by extrapolation to zero time.

## Results

### *Purine Derivatives As Substrates of Milk Xanthine Oxidase*

**Dioxypterines**—We have confirmed the results of Coombs (1) that xanthine and 6,8-dioxypterine react at about the same rate (Table II). This is true over the whole pH range studied (see below). However, 2,8-dioxypterine is oxidized at a much lower rate, indicating that a certain

mutual relationship must exist for the enzymatic attack at positions 2 and 8 in the purine nucleus. The reaction in the first two cases is linear, until about 40 per cent conversion, whereafter the rate decreases.

*Monooxypurines*—In this group, *hypoxanthine* exceeds the other two isomers by far (Table II). The figures of Table II show that the first oxidation step of hypoxanthine must be slower than the second one. Since 2,6- and 6,8-dioxypurines are converted into uric acid at the same rate, it remains open whether the intermediate is exclusively xanthine, as is generally assumed, or whether 6,8-dioxypurine may also be involved.

TABLE II

*Relative Rates of Oxidation of Purine Derivatives by Xanthine Oxidase*

Xanthine served as a reference compound. Substrate concentration  $10^{-4}$  M. Temperature 21°, pH = 8.0. The column "Oxidative pathway" indicates the order in which the oxidizable positions were attacked by the enzyme.

Substrate	Product measured	Oxidative pathway	Relative rate with	
			Milk $\lambda$ O	Liver $\lambda$ O
Xanthine	Uric acid		1.0	1.0
1-Methylxanthine	1-Methyluric acid		0.45	1.0
6,8-Dioxypurine	Uric acid		1.0	1.1
Hypoxanthine	Xanthine, uric acid (6,8-dioxypurine?)	→ 2,6 → 2,6,8	0.7	0.7
Purine	Uric acid	→ 6 → 2,6 → 2,6,8	0.2	0.15
2-Oxypurine	2,8-Dioxypurine	→ 2,8 → 2,6,8	0.16	0.04
8-Oxypurine	"	→ 2,8 → 2,6,8	0.015	0.002
"	Uric acid		0.002	0.0005
2,8-Dioxypurine	" "		0.002	0.0005

In order to decide this point, the experiment described in Table III was carried out at pH 5.5, at which the reaction rates are sufficiently low to permit detection of the intermediates spectrophotometrically. Readings were then taken at the following wave lengths (see Fig. 3). At 249  $m\mu$ , the isosbestic point of hypoxanthine and 6,8-dioxypurine, any decrease in optical density represents formation of xanthine or uric acid or both. At 262.5  $m\mu$ , the isosbestic point of hypoxanthine and xanthine, any increase in optical density is due to 6,8-dioxypurine and any decrease to uric acid. At 270  $m\mu$ , the isosbestic point of uric acid and hypoxanthine on the one hand and of xanthine and 6,8-dioxypurine on the other, any increase in optical density represents the sum of the latter two derivatives. At 300  $m\mu$  mainly uric acid is measured, since 6,8-dioxypurine makes only a minor contribution.

Inspection of Table III shows the following (a) Hypoxanthine is continuously being used up (b) A very slight initial rise appears at 262.5  $m\mu$ , while formation of uric acid is still negligible This may be taken as an indication that some 6,8-dioxypurine appears during the oxidation of hypoxanthine However, the effect is too small to give conclusive evidence about this point (c) The readings at 270  $m\mu$  show that, between the 2nd and 10th minute, xanthine concentration reaches a steady state in which formation and degradation of this intermediate are balanced (d) Uric acid formation lags behind for the first few minutes and becomes appreciable after about 4 minutes These observations confirm that the

TABLE III  
*Intermediates in Oxidation of Hypoxanthine to Uric Acid*

Enzyme 1 200, hypoxanthine  $0.5 \times 10^{-4} M$ , 0.1 M acetate buffer of pH 5.5, temperature 21°

Time	249 $m\mu$	262.5 $m\mu$	270 $m\mu$	300 $m\mu$
<i>hrs</i>				
0.5	0.55	0.49	0.36	0.055
1.3	0.51	0.50	0.38	0.06
2	0.48	0.50	0.42	0.075
3	0.45	0.49	0.425	0.09
4	0.43	0.48	0.43	0.16
6	0.40	0.43	0.43	0.22
10	0.37	0.37	0.41	0.32
15	0.36	0.32	0.38	0.39
20	0.36	0.30	0.36	0.40
25	0.36	0.30	0.36	0.40

oxidation of hypoxanthine passes almost exclusively through xanthine as intermediate

Oxidation of 8-oxypurine may give either 2,8- or 6,8-dioxypurine, either of which is converted into uric acid Booth (2) concluded from kinetic measurements that the 2,8 isomer must be the intermediate The spectrophotometric method permits us to establish this point beyond doubt At 320  $m\mu$ , 8-oxypurine, 6,8-dioxypurine, and uric acid do not show absorption Therefore, any increase in optical density at this wave length measures exclusively 2,8-dioxypurine Simultaneously, at 295  $m\mu$  (the isosbestic point of 8-oxypurine and 2,8-dioxypurines) the formation of uric acid can be followed (6,8-dioxypurine showing only weak absorption at this wave length) (see Fig. 1) Only very small amounts of the latter are formed during the usual observation time (1 hour), the rate for conversion of 8-oxypurine into uric acid being only one-fifth of that for the

reaction 8-oxypurine  $\rightarrow$  2,8-dioxypurine. On the other hand, the rate for the oxidation of 6,8-dioxypurine is about 500 times faster than that of the 2,8 isomer, hence measurable quantities of uric acid should appear in the early part of the reaction, if the alternative pathway 8-oxypurine  $\rightarrow$

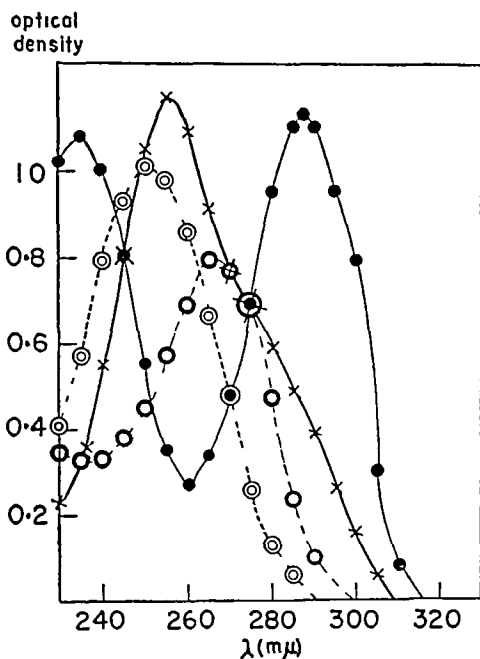
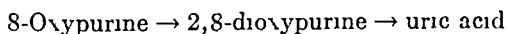
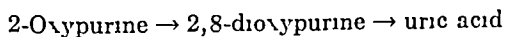


FIG 3 Absorption spectra of various purine derivatives at pH 5.5. Concentration used,  $10^{-4}$  M.  $\odot$ , hypoxanthine,  $\odot$ , xanthine,  $\times$ , 6,8-dioxypurine,  $\bullet$ , uric acid. The isosbestic points used in this paper are 249  $m\mu$ , hypoxanthine and 6,8-dioxypurine, 262.5  $m\mu$ , hypoxanthine and xanthine, 270  $m\mu$ , hypoxanthine and uric acid, xanthine and 6,8-dioxypurine.

6,8-dioxypurine were quantitatively important. The *main* route of oxidation is thus the one indicated by Booth



The rate of oxidation of 2-oxypurine into 2,8-dioxypurine is 30 times faster than conversion of the latter into uric acid (see Table II). At 317  $m\mu$ , no change in optical density was observed during the first  $\frac{1}{2}$  hour, indicating that the sum of 2-oxypurine plus 2,8-dioxypurine remained constant. Therefore, the intermediate accumulates, without any measurable quantities of uric acid, during the first  $\frac{1}{2}$  hour, and thus permits unequivocal analysis of the enzymatic pathway as follows



The alternative route via 2,6-dioxypurine is definitely excluded. The

experiments on monooxypurines again demonstrate the mutual relationship between carbons 2 and 8 in the purine skeleton. Previous oxidation at either position directs further oxidative attack to the other partner of this pair, excluding carbon 6.

*Purine*—This substrate is converted into uric acid at about one-third the speed of hypoxanthine (Table II). If 2- or 8-oxypurine would participate in the first oxidation step, 2,8-dioxypurine should accumulate (as shown under "Monooxypurines"). No trace of a substance absorbing

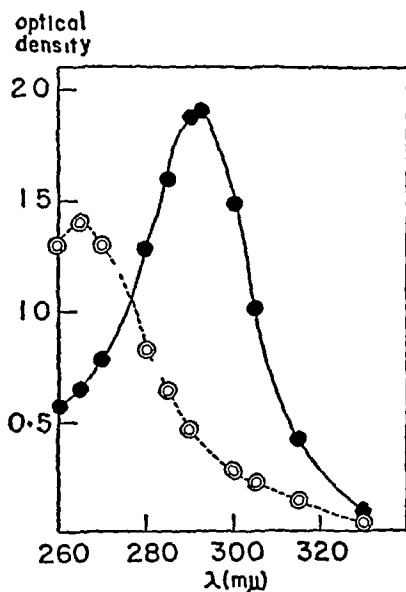
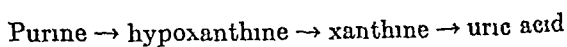


Fig. 4. Enzymatic oxidation of purine into uric acid. ○, initial absorption spectrum of purine; ●, absorption spectrum after enzymatic digestion for 45 minutes. Substrate concentration,  $1.75 \times 10^{-4}$  M, pH 8.0, enzyme dilution 1:20. Each curve represents the spectrum measured against enzyme and buffer, without substrate, as blank.

at 320 mμ was, however, found. In Fig. 4, an experiment is shown in which a concentrated enzyme solution (1:20) produced about 95 per cent conversion during 45 minutes. The final spectrum of the reaction mixture, obtained after subtracting the "blank" value of the control, containing enzyme without substrate, indicates pure uric acid. These observations establish the oxidative pathway as follows:



*Methylated Purine Derivatives*—Like 7-methylhypoxanthine (2) the 1-methyl derivative also was found to be inactive.

The successful synthesis of 1-methylxanthine (5) enabled us to test this substrate. It is oxidized by milk xanthine oxidase at one-half the

rate of the mother substance. Since uric acid and its 1-methyl derivative cannot be distinguished spectrophotometrically, identification of the oxidation product as 1-methyluric acid was accomplished by paper chromatography (5). A mixture of 1-methylxanthine (0.1 ml = 100  $\gamma$ ), phosphate buffer (0.1 ml), undiluted XO (0.1 ml), and a drop of chloroform was incubated at 34° for 24 hours and 0.06 ml (20  $\gamma$ ) was spotted on paper, together with uric acid, 1-methyluric acid, and 1-methylxanthine for comparison. The descending chromatogram was developed with a

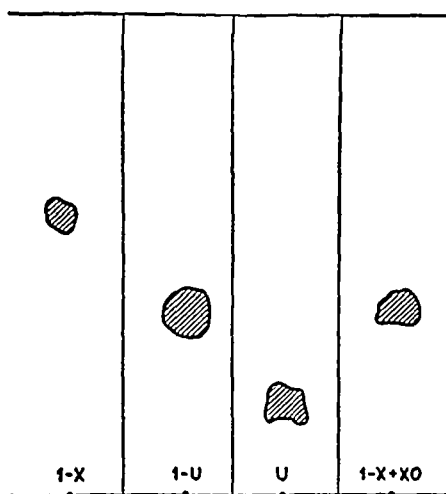


FIG 5 Descending paper chromatogram of the oxidation product of 1-methylxanthine by milk xanthine oxidase. Enzyme dilution 1:3, substrate 20  $\gamma$  per ml, pH 8.0. Solvent used for development, ethanol-water-acetic acid (85:10:5). The spots were made visible by the mercury-diphenylcarbazone method, described previously (5). The following  $R_F$  values were obtained: 1-methylxanthine (1-X), 0.58; 1-methyluric acid (1-U), 0.38; uric acid (U), 0.2; enzymatic oxidation product of 1-X, 0.38.

mixture of ethanol-water-acetic acid (85:10:5 volumes). The result, given in Fig 5, identifies the reaction product unequivocally.

None of the other mono-, di-, and trimethylxanthines was attacked by purified xanthine oxidase, as observed already by previous investigators (1, 9).

#### *Substrate Specificity of Human Liver Xanthine Oxidase*

In view of the interest in the human metabolism of substituted xanthines, it appeared necessary to establish the specificity of the xanthine oxidase of human organs. As a representative example, the liver enzyme, which has been studied previously by Richert and Westerfeld (10), was purified



and tested. The results were in all respects analogous to those obtained with the enzyme from cow's milk (see Table II)

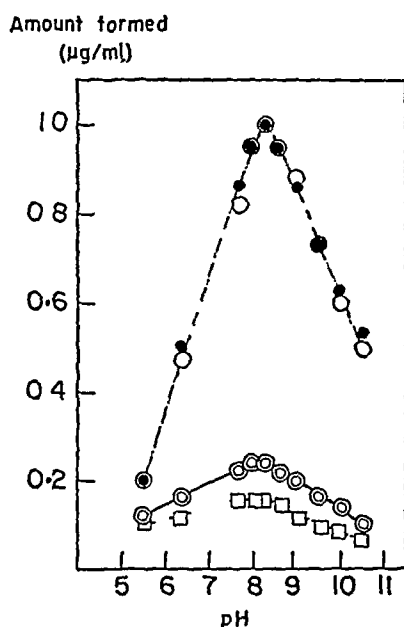


Fig 6 Rate of oxidation of various substrates by xanthine oxidase as function of pH. All points in this graph are calculated for an enzyme dilution of 1/400. However, the actual experiments with purine were carried out with XO 1/80 and those with 2-oxypurine with XO 1/40. Substrate concentration  $10^{-4}$  M. ●, xanthine, ○, 6,8-dioxypurine, ⊙, purine. For these three substrates the ordinate represents uric acid formed (microgram per ml). □, 2-oxypurine, ordinate, 2,8 dioxypurine formed.

TABLE IV  
*pH Variation of Enzymatic Rates*

Substance	pK	Ionization at pH 8.3	Reference
		<i>per cent</i>	
Purine	8.9	20	Albert and Brown (12)
2-Oxypurine	8.5	40	" " " (12)
6,8-Dioxypurine	8.0	66	Present authors, unpublished
Xanthine	7.7	80	" " (13)

Because of the low activity of our purified preparation, rate figures less than 0.1 are less reliable than those obtained with the milk enzyme. It is of interest that 1-methylxanthine is oxidized as fast as xanthine itself.

#### *Influence of pH Changes on Activity of Milk Xanthine Oxidase*

Since the most active substrates of XO differ in their pK values, the possibility has to be considered that their different rates might result,

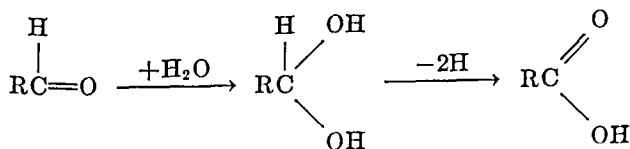
at least in part, from the different ratios of anions to uncharged molecules at a given pH. If this should be the case, the pH-activity curves should give information as to which form has the greater affinity to the enzyme and thus should shed light on the mechanism of the reaction. The results obtained with some substrates of sufficiently high activity (Fig. 6) demonstrate that the positions of the pH maxima or the slopes of the curves are very similar and agree with the results of Krebs and Norris (11). The curves in Fig. 6 cannot be extended beyond pH 10.5, since we have found that the enzyme undergoes irreversible destruction at about pH 11.0. These observations have to be considered in the light of the fact that pK values and thus degree of dissociation at optimal pH differ considerably (Table IV). It can be concluded that the pH variation of enzymatic rates reflects essentially changes taking place in the active surface.

#### DISCUSSION

The present experiments establish the main oxidative pathways in the purine series unequivocally and demonstrate that the rate and direction of oxidative attack depend on the position of oxygen introduced previously. The first attack in purine itself is at carbon 6. With hypoxanthine, the next step is directed towards carbon 2. But positions 2 and 8 form a closely related pair, hence oxidation at one of these carbons leads always to attack at the other partner in the next step.

All purine derivatives, tested in this investigation, can be divided into three groups with respect to their activity against mammalian xanthine oxidase. (1) *Substrates with a rate of oxidation comparable to xanthine* are 1-methylxanthine, 6,8-dioxypurine, hypoxanthine, and purine. (2) *Substrates which are attacked at about one-tenth to one-ten thousandth the rate of xanthine* are 2- and 8-oxypurines and 2,8-dioxypurine. (3) *Purine derivatives which are not attacked at a measurable rate* are 1- and 7-methylhypoxanthines, 3-, 7-, and 9-methylxanthines, and xanthosine, all dimethylated xanthines, and caffeine.

Our results enable us to draw certain conclusions about the way in which a substrate attaches itself to the active center and about the mechanism of dehydrogenation by xanthine oxidase. We shall start this discussion by comparing purine oxidation with the conversion of aldehydes into acids, a reaction brought about by a very similar enzyme. Here the following scheme applies:



In accordance, we may formulate the conversion of purine into hypoxan-

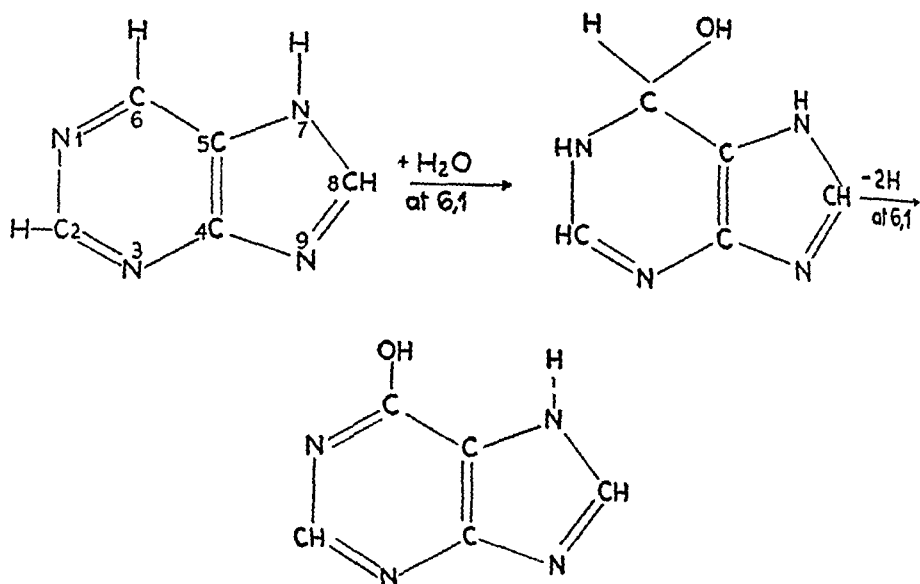


DIAGRAM 1 Formation of lactim of hypoxanthine

thine as shown in the accompanying scheme (Diagram 1) The C=\ bond in the heterocyclic nucleus behaves like the carbonyl group of an aldehyde. Dehydrogenation at position 6,1 directs further attack to the adjacent double bond 2,3, where again hydration and dehydrogenation

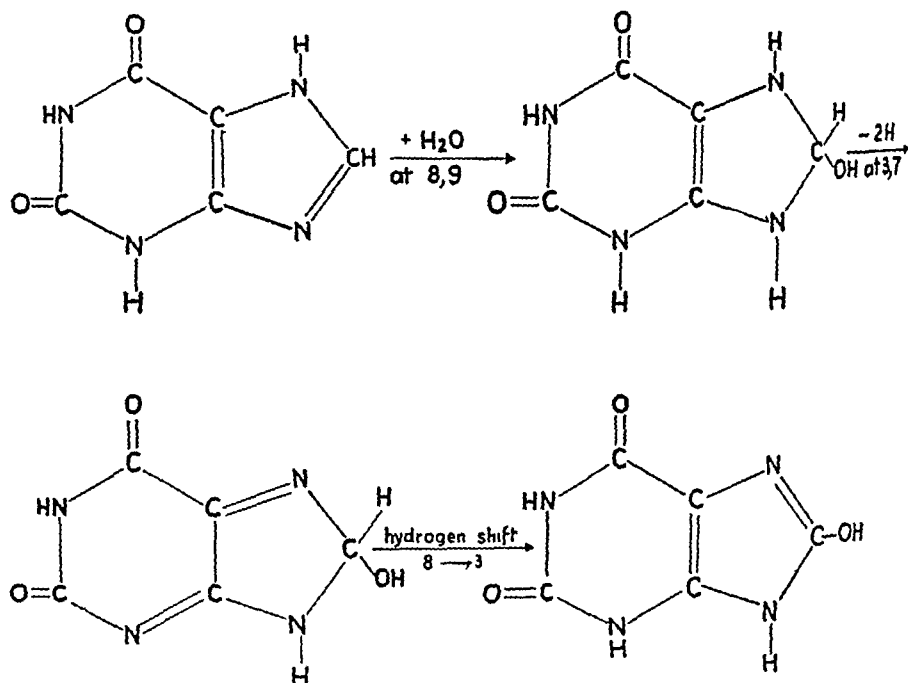


DIAGRAM 2 Formation of 8-lactim of uric acid

take place This reaction is prevented, when either N-1 or N-7 is methylated Thus we may conclude that the enzyme attaches itself not only to position 2,3 in hypoxanthine, but simultaneously to N-1 and N-7 A multiple point attachment of xanthine oxidase to its substrates is indicated

On the other hand, oxidation of xanthine is prevented by substitution at N-3, N-7, or N-9, but proceeds normally with the N-1-methyl derivative We conclude that the mode of attachment *changes* from hypoxanthine, in which N-1, N-3, and N-7 are involved, to xanthine, in which N-3, N-7, and N-9 must be free for combination with the enzyme This change

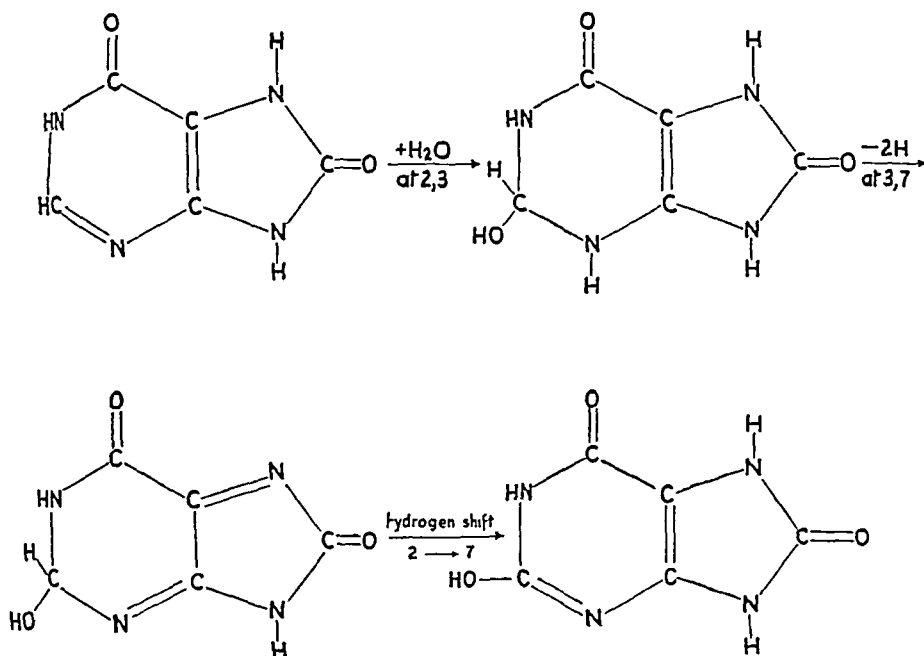


DIAGRAM 3 Formation of 2-lactim of uric acid

makes it probable that dehydrogenation of xanthine proceeds as indicated in Diagram 2 According to the scheme, the decisive step is dehydrogenation of the grouping  $\text{HN}(3)-\text{C}(4)=\text{C}(5)-\text{N}(7)\text{H}$  to the corresponding dienic system, which is analogous to the reactive portion in the molecule of riboflavin in the prosthetic group of xanthine oxidase It becomes apparent at once that oxidation of 6,8-dioxypurine is in complete harmony with this view, the accompanying steps (Diagram 3) being involved In the same way, the mutual relationship between 2- and 8-oxypurines, which are both converted into 2,8-dioxypurine, is easily explained It should be noted that a similar mechanism may also apply to the oxidation of hypoxanthine or purine, e.g., the hydrated form of hypoxanthine may undergo dehydrogenation at N-3 and N-7 to give the intermediate (Diagram 4),

2 A peculiar relationship between position 2 and 8 in the purine ring has been found. Oxidation at either position directs further attack to the other partner of this pair.

3 Xanthine oxidases from cow's milk or human liver show identical substrate specificity. Among all substituted xanthines only the 1-methyl derivative is attacked by the enzyme and converted into 1-methyluric acid.

4 A hypothesis is advanced for multiple point attachment of purines to xanthine oxidase and for the mechanism of dehydrogenation.

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# THE LOW TEMPERATURE SPECTRA OF HEMOPROTEINS\*

## I APPARATUS AND ITS APPLICATION TO A STUDY OF CYTOCHROME *c*

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The application of low temperature spectroscopy to biological problems has included the study of porphyrins (1, 2), chlorophyll (3-6), amino acids (7, 8), nucleotides (9), and hemoproteins (10). The changes described in the absorption spectra of pigments cooled to  $-190^{\circ}$  include (a) a pronounced sharpening of some of the absorption bands, (b) a shift of the absorption maxima, (c) a splitting of some of the absorption bands, and (d) an intensification of these bands.

The studies on the spectral properties of porphyrins, chlorophyll, amino acids, and nucleotides have been carried out with organic solvents which vitrify to glass-like solids on cooling. Such solvents give clear solids which can be investigated spectrophotometrically (2, 6). The use of organic solvents with hemoproteins, however, is impractical because of the low solubility and possible denaturation of the protein. Keilm and Hartree (10) therefore devised a spectroscopic method, with glycerol as solvent, which permitted them to investigate the effect of low temperature upon the visible absorption bands of hemoproteins. They recognized that devitrification of mixtures containing such a solvent (glycerol) in the proper concentration resulted in the development of a turbid microcrystalline state which caused a marked intensification of the absorption bands (11). The visual spectroscopic studies by Keilm and Hartree showed the advantages of low temperature spectroscopy for distinguishing cytochrome pigments with nearly identical absorption maxima. Indeed, their systematic study of a variety of biological materials revealed the presence of a previously undiscovered hemoprotein, which they have called cytochrome  $c_1$  or  $c$  (10, 12).

This paper describes a technique for recording spectrophotometrically the absorption bands of samples of hemoproteins cooled in liquid air to  $-190^{\circ}$ . The method described combines that employed by Keilm and

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A preliminary report of this work has been published (*Biochim et biophys acta*, **19**, 184 (1956)).

Hartree (10) for their visual study and the spectrophotometric methods described by others (2, 6) for the study of chlorophyll and porphyrins. The present procedure has the advantage of plotting automatically the absorption spectra of turbid suspensions of hemoproteins cooled to low temperatures. This is accomplished by the use of the sensitive wave length scanning spectrophotometer developed by Chance and his coworkers (13-16). The results are presented for reduced cytochrome *c*. The low temperature spectra of cytochrome *c* prepared from heart muscle, yeast, and *Rhodospirillum rubrum* are compared.

### Apparatus

Initial attempts at recording spectra of precooled samples of hemoproteins with the conventional cuvette holder of the spectrophotometer gave unsatisfactory results. These preliminary studies showed that it would be necessary to devise an adapter which could contain liquid air. The adapter, shown in Fig. 1 with the photomultiplier attached, consists of a light-tight box containing a 450 ml. unsilvered Dewar flask and a movable cuvette holder. Similar adapters have been constructed by others (2, 6) and used with Beckman model DU spectrophotometers and Cary recording spectrophotometers for the study of porphyrins and chlorophylls. The use of such an adapter was found necessary to maintain the temperature of the sample, to prevent the formation of frost on the surfaces of the cuvettes, and to eliminate fog, which results if the cold cuvettes are brought in contact with the moisture in air at room temperature.

The adapter is attached to a Bausch and Lomb diffraction-grating monochromator in place of the standard cuvette holder. The beam of incident light from the monochromator passes through the Dewar flask and cuvettes to a Dumont K1234 end-on photomultiplier and is deflected by a 30 cps oscillating mirror so that it passes alternately through one cuvette and then the other. The per cent transmission was converted into optical density by a logarithmic network (15), and the difference in optical density between the sample and the reference cuvette was plotted automatically as a function of wave length on a Leeds and Northrup Speedomax recorder. The magnitude of the absorption bands may be determined from the "optical density or absorbancy increment index." The optical density or absorbancy increment is equal to  $\log I_s/I_b$  for a cuvette of a given optical depth, where  $I_s$  is the intensity of the light transmitted through the sample and  $I_b$  is the intensity of the light transmitted through the reference cuvette.

Fig. 2 illustrates in detail the holder and cuvettes that were used. The cuvettes were constructed of sheet Plexiglas with a horizontal cross section in the shape of the letter H with an optical depth (path length) of 1 mm.

The design of the cuvettes and the holder allowed for rapid and uniform cooling of small amounts (0.5 ml) of material. When placed in the mov-

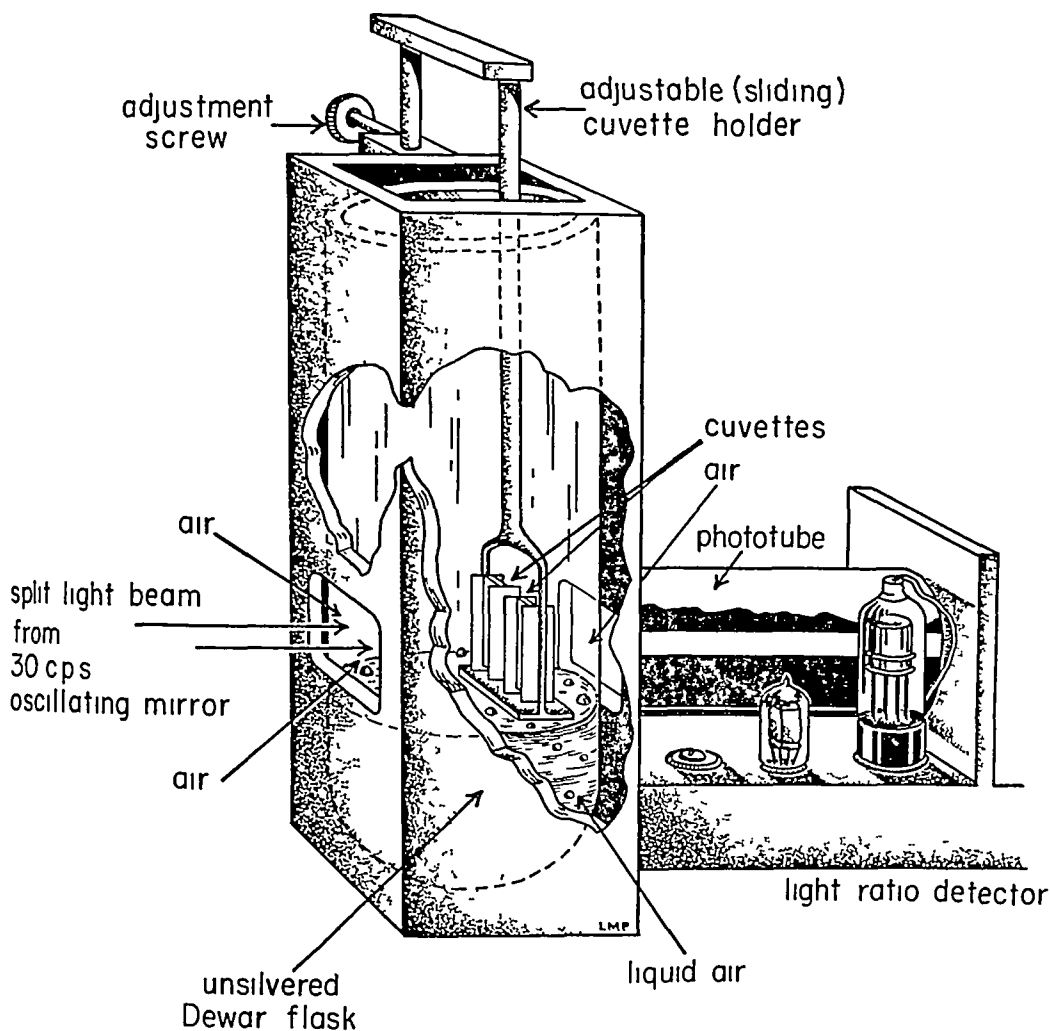


FIG 1 Schematic drawing of adapter used in obtaining spectra of samples cooled to low temperatures. Monochromatic light is split into two beams by a 30 c p s oscillating mirror. The split beams of incident light pass through the unsilvered Dewar flask to the sample and reference cuvettes. Transmitted light passing out of the Dewar flask activates the photomultiplier located in the light ratio detector. The cuvettes are placed in a cuvette holder attached to a sliding rod so that the cuvettes may be raised or lowered as desired. A Bakelite lid for the adapter is not shown.

able holder, two cuvettes, one containing the sample to be analyzed and the other an appropriate blank, could be plunged into liquid air, raised and aligned in the light beam for recording the spectrum, or removed from the Dewar flask.



Since the sample is not immersed in the liquid air during the recording of a spectrum, it was necessary to determine the change in temperature of the contents of the cold cuvettes with time, when these cuvettes were placed 2 to 3 cm above the level of liquid air. Measurements of temperature were made with a non-constantan thermocouple immersed in the

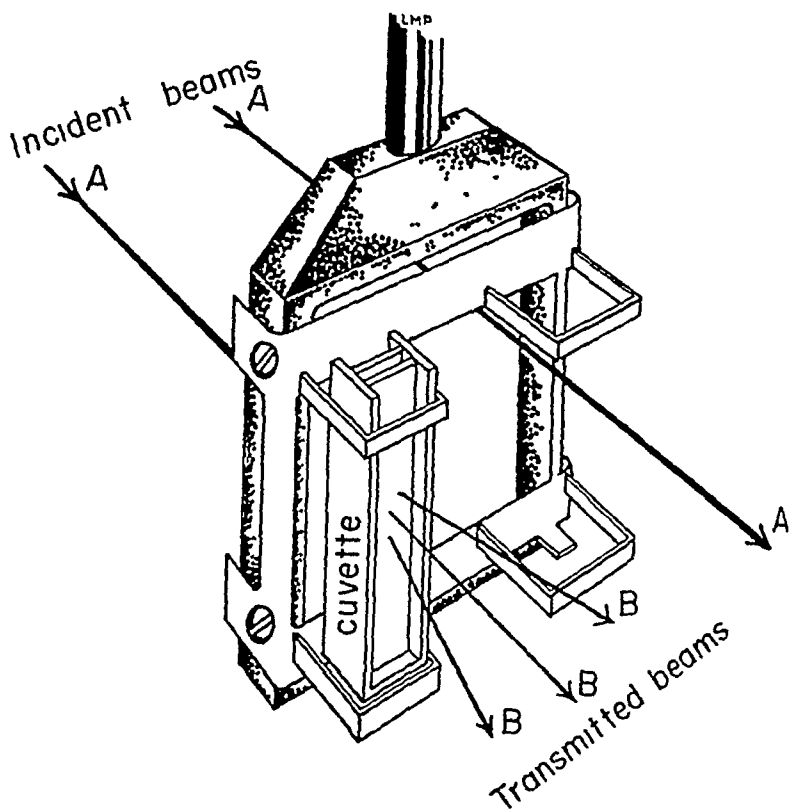


Fig 2 A detailed drawing of cuvette and holder. The cuvettes are constructed of sheet Plexiglas and have a 1 mm optical depth. The beams of incident light (A) from the monochromator pass alternately through the contents of the cold cuvette to give the transmitted light beams (B). One cuvette has been omitted to show the construction of the holder.

contents of the cuvette. The change in temperature during a typical recording of 30 to 40 seconds duration (Fig 3) would be from  $-189^{\circ}$  to about  $-175^{\circ}$ .

### Materials

Cytochrome *c* of heart muscle was purchased from the Sigma Chemical Company and purified on Amberlite IRC-50 ion exchange resin by the method of Margolash (17). Yeast cytochrome *c* was prepared as described

elsewhere,<sup>1</sup> and cytochrome  $c_2$  was prepared<sup>2</sup> from *R. rubrum* by a modification of the method of Vernon and Kamen (18)

### EXPERIMENTAL

*Sharpening and Splitting of Absorption Bands of Cytochrome c*—Purified cytochrome  $c$  of heart muscle was diluted with 0.1 M phosphate buffer, pH 7.4, and sodium dithionite was added as a reducing agent. The solution of reduced cytochrome  $c$  was then diluted with an equal volume of glycerol

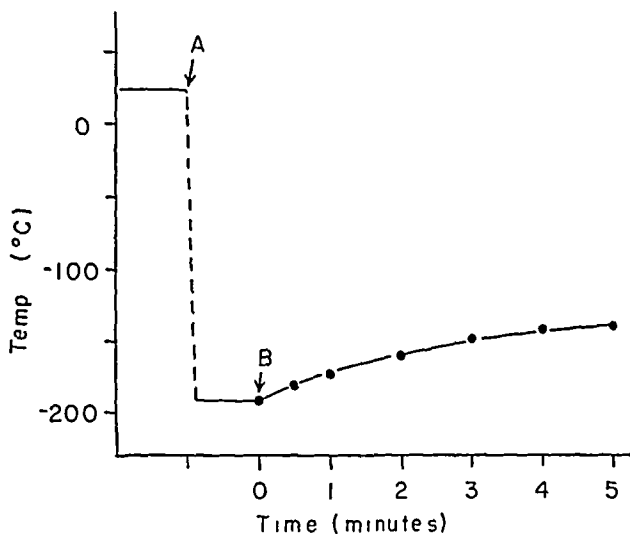


FIG. 3 The change in temperature of the sample after cooling in liquid air. Measurements of temperature were made with an iron-constantan thermocouple inserted into the contents of a cuvette. The cuvette contained a mixture of 50 per cent glycerol 50 per cent 0.1 M phosphate buffer, pH 7.4. The cuvette with the thermocouple inserted was cooled in liquid air (A) and then raised 2 to 3 cm above the level of liquid air (B) into the light beams.

and the spectrum was recorded at room temperature, a mixture of equal volumes of glycerol and phosphate buffer being used in the reference cuvette. The spectrum obtained (Fig. 4) shows the expected maxima for the absorption bands of reduced cytochrome  $c$  at 550, 520, and 415  $m\mu$ . The maximum at 550  $m\mu$  of reduced cytochrome  $c$  has been taken as the reference (a calibration point) for all subsequent wave length determinations.

When liquid air is placed in the Dewar flask and the contents of the cuvettes are rapidly cooled to  $-189^\circ$ , the glycerol mixtures vitrify to a glass-like state (Condition I), but many cracks develop when the mixtures

<sup>1</sup> Estabrook, R. W., manuscript in preparation.

<sup>2</sup> Prepared by Dr. Lucile Smith of this laboratory.

are cooled in such a manner. The spectrum of the sample cooled in liquid air (Condition I) shows a sharpening of the visible absorption bands with a slight displacement of the maxima to shorter wave lengths as well as a splitting of the  $\alpha$ - and  $\beta$ -bands. The intensification of the  $\alpha$ - and  $\beta$ -bands observed here may be due in part to contraction of the solvent during the rapid cooling in liquid air. The  $\alpha$ -band, which at room temperature ap

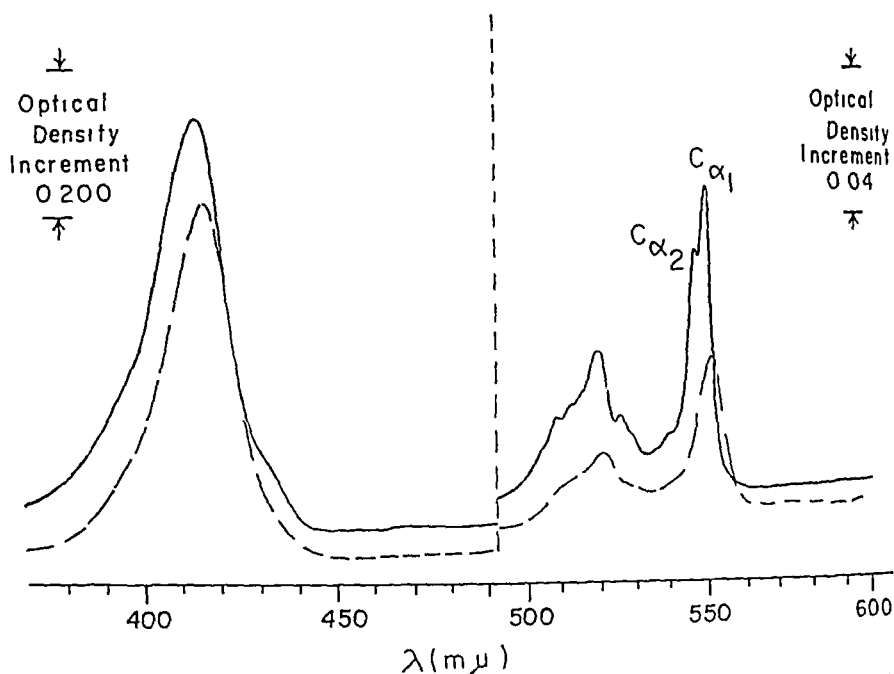


FIG. 4. Comparison of the spectrum of reduced cytochrome *c* at room temperature and at  $-189^{\circ}$ . The dashed curve represents the spectrum of the sample at room temperature, the solid curve is the spectrum of the sample after cooling in liquid air (Condition I). The sample cuvette contained a mixture of 0.2 ml of  $2 \times 10^{-4}$  M cytochrome *c*, 0.3 ml of 0.1 M phosphate buffer, pH 7.4, a few crystals of sodium dithionite, and 0.5 ml of glycerol. The reference cuvette contained a mixture of 50 per cent glycerol and 50 per cent 0.1 M phosphate buffer, pH 7.4. The effective band width was 0.6  $m\mu$ , the optical depth of the cuvette was 1 mm.

pears as a single absorption band with a maximum at 550  $m\mu$ , is resolved into three bands with maxima at about 549, 546, and 538  $m\mu$ . The original  $\beta$ -band is split into a variety of bands at low temperatures. The major bands in the  $\beta$  region have maxima at about 525, 519, and 508  $m\mu$  with indications of bands at about 529, 515, 512, and 503  $m\mu$ . The  $\gamma$  band at low temperatures has a maximum at about 414  $m\mu$  with a shoulder at about 432  $m\mu$ . In subsequent discussions, the bands of reduced cytochrome *c* of heart muscle, which at low temperatures have maxima at about 549, 546, and 538  $m\mu$ , will be designated  $c_{\alpha_1}$ ,  $c_{\alpha_2}$ , and  $c_{\alpha_3}$ , respectively.

*Intensification of Absorption Bands*—When the cold cuvettes were removed from the Dewar flask, the two changes described by Keilin and Hartree (10, 11) were observed as the temperature of the sample and the blank was allowed to rise. In reflected light, at about  $-75^{\circ}$ , a faint opalescence could be seen to pass down through the contents of both cuvettes. The same change observed in transmitted light was seen as a coloration similar to sunglow. The second phenomenon occurred at  $-55^{\circ}$ , where the contents of both cuvettes were observed to become much more turbid. When

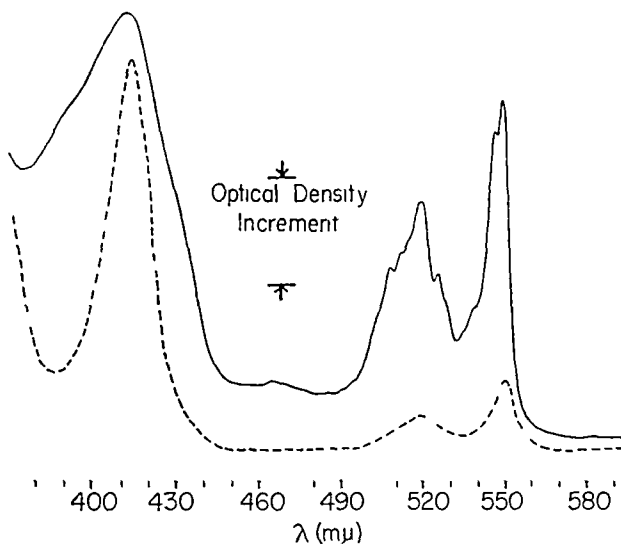


Fig 5 Absorption spectra of reduced cytochrome *c*. The dotted curve represents the spectrum obtained when the sample is at room temperature, the solid curve is at liquid air temperatures (Condition II). The sample cuvette contained 0.1 ml of  $2 \times 10^{-4}$  M cytochrome *c*, 0.4 ml of 0.1 M phosphate buffer, pH 7.4, a few crystals of sodium dithionite, and 0.5 ml of glycerol. The reference cuvette was as described for Fig 4. The effective band width, 0.5 mμ, optical depth of cuvettes, 1 mm.

the turbidity appeared to be uniform throughout the contents of the cuvettes, they were again placed in liquid air and cooled to  $-189^{\circ}$ . This state is called Condition II. The turbidity "formed," as described above, has been attributed by Keilin and Hartree (11, 19) to the formation of microcrystals during devitrification. This turbidity remains if the samples are again cooled in liquid air. Although the difference spectrum of these turbid mixtures (Fig 5) shows that the position of the bands is the same as those described above for the sample in Condition I, a pronounced intensification by the  $\alpha$ - and  $\beta$ -bands was obtained. The spectrum of the same sample, as obtained when it was at room temperature, is included in Fig 5 for comparison with the low temperature spectrum.

The low temperature spectrum obtained when cytochrome *c* of heart

muscle is reduced enzymatically by the succinoxidase system of a heart muscle preparation shows maxima identical with that obtained when sodium dithionite was used as a reducing agent. Alkaline incubation of the cytochrome *c*, prior to reduction by dithionite, obliterates irreversibly the  $c_{\alpha_2}$  band at  $546 m\mu$  (10)

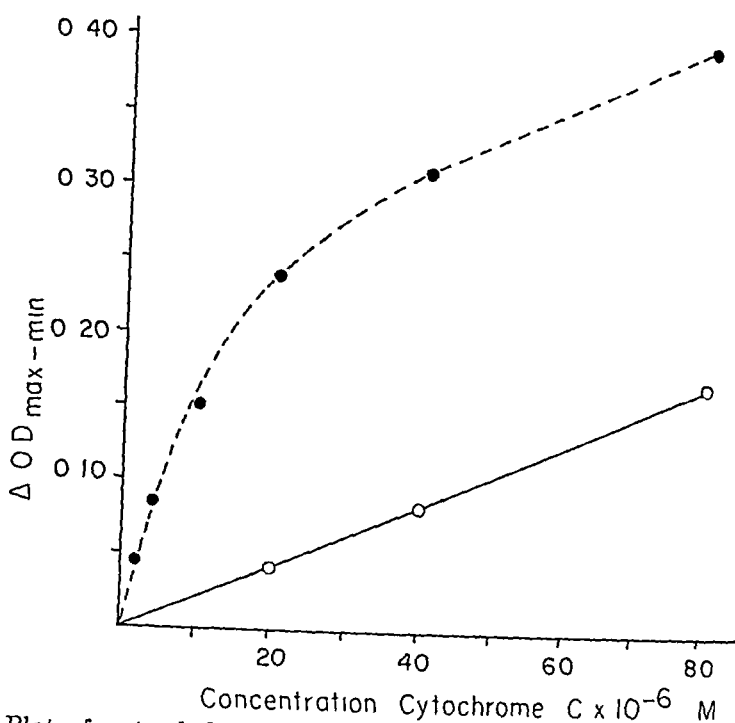


Fig 6 Plot of optical density difference against the concentration of reduced cytochrome *c*. The solid curve represents the  $\Delta OD_{\max-\min}$  of the  $\alpha$  maximum at  $550 m\mu$  and the minimum at about  $540 m\mu$  of the sample at room temperature. The dashed curve is that of the  $\Delta OD_{\max-\min}$  of the  $c_{\alpha_1}$  maximum and the minimum at about  $535 m\mu$  after cooling in liquid air to Condition II. The reference cuvette contained a mixture of 50 per cent glycerol-50 per cent 0.1 M phosphate buffer, pH 7.4. The sample cuvette contained a mixture of 0.5 ml of variable concentrations of cytochrome *c* in 0.1 M phosphate buffer, pH 7.4, a few crystals of sodium dithionite, and 0.5 ml of glycerol. The optical depth of cuvette was 1 mm, the effective band width was  $0.6 m\mu$ .

The spectrum recorded when the material is in Condition II (turbid state) is the spectrum of the contents of the sample cuvette minus that of the reference cuvette (the total optical density when measured *versus* air is about 1.25). Since the spectra presented are corrected for a reference of about equal turbidity, any quantitative measure of the pigment concentration must be expressed in terms of optical density differences between an absorption band maximum and a corresponding minimum. For cytochrome *c*, the optical density (O D) difference between the  $\alpha$  maximum and the minimum at about  $540 m\mu$  will be termed  $\Delta OD_{\max-\min}$ . The

ratio for the  $\Delta OD_{\max-\min}$  may be used as a measure of the intensification of the  $\alpha$ -band when the room temperature and Condition II spectra are compared. For the spectra in Fig 5, this ratio is about 4 and depends upon the concentrations of reduced cytochrome *c* employed. The relationship between the hemoprotein concentration, however, and the  $\Delta OD_{\max-\min}$  (Fig 6) was not linear. The intensification of the  $\alpha$ -band appears greater for more dilute solutions of the reduced pigment. An examination of the Soret band of reduced cytochrome *c* (Fig 5) shows that there is only a small intensification of this absorption band. The intensification of the  $\alpha$ -band of reduced cytochrome *c*, which is bound to particulate material, is, however, greater.<sup>3</sup>

The turbidity which occurs when the sample is devitrified appears only when the mixture contains about 50 per cent glycerol. If less than 45 per cent or more than 55 per cent glycerol is present, the sample simply melts upon warming and the turbid state is never achieved. The presence of small amounts of alcohol or high salt concentrations also prevents the formation of the turbidity characteristic of Condition II.

*Displacement of Absorption Bands*—The above results for the sharpening, splitting, and intensification of the absorption bands agree well with the visual observations of Keilin and Hartree (10). Our measurements of the displacement of absorption maxima at low temperatures do not agree, however, with those reported by Keilin and Hartree. They have stated that, "when a solution of reduced cytochrome *c* is cooled to the temperature of liquid air, its absorption bands are intensified about six times and shifted by about 10  $m\mu$  toward the blue." Fig 7 compares the  $\alpha$ - and  $\beta$ -bands of reduced cytochrome *c* at both room and low temperatures, and shows that the displacement of the  $c_{\alpha_1}$  absorption maximum is only about 1 to 1.5  $m\mu$ . A partial explanation for this discrepancy is offered by Hartree (11), who indicates that a 6 to 10  $m\mu$  band shift occurs when solutions of hematin derivatives are cooled in liquid air, but that "freezing of the solvent may lead to aggregation and to a superimposed shift, of the same order of magnitude, in the opposite direction." Recorded spectra of samples of reduced cytochrome *c*, cooled in phosphate buffer without glycerol, or to Conditions I or II as described above, show that the  $c_{\alpha_1}$  band of cytochrome *c* always has a maximum at about 548 to 549  $m\mu$ . The cause of this discrepancy will require further study in order to determine the variance between the results of visual observations and the spectrophotometric method.

*Spectrum of Samples Cooled to  $-72^\circ$* —The formation of the microcrystalline turbid state (Condition II) may also be achieved in another way. If a sample of reduced cytochrome *c*, suspended in 50 per cent glycerol

<sup>3</sup> Estabrook, R. W., and Mackler, B., manuscript in preparation.

is cooled to  $-72^{\circ}$  in an alcohol-dry ice mixture, a similar turbidity is slowly formed (Condition III) Fig 8 shows the spectrum of a sample treated

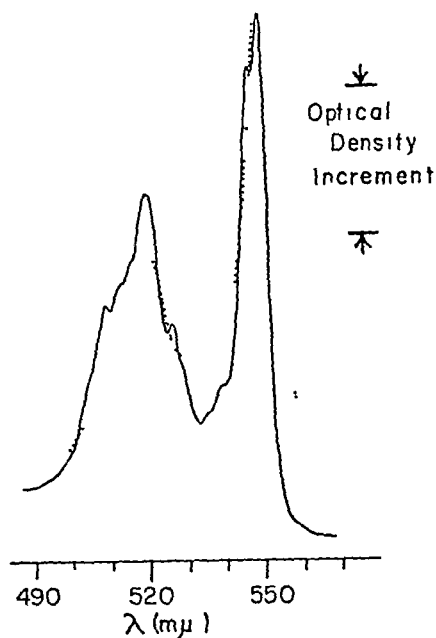


FIG 7

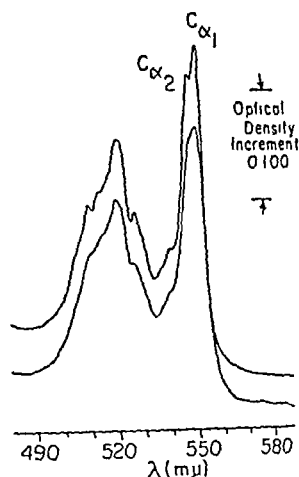


FIG 8

FIG 7 The displacement of absorption bands at low temperatures The dotted curve represents the spectrum of reduced cytochrome *c* at room temperature, the solid curve is the spectrum of the same sample cooled in liquid air (Condition II) The optical density increment was 0.02 and 0.10, respectively The sample cuvette contained a mixture of 0.1 ml of  $2 \times 10^{-4}$  M cytochrome *c*, 0.4 ml of 0.1 M phosphate buffer, pH 7.4, a few crystals of sodium dithionite, and 0.5 ml of glycerol The reference cuvette contained a mixture of 50 per cent glycerol and 50 per cent 0.1 M phosphate buffer, pH 7.4 The effective band width was 0.6  $m\mu$ , the optical depth of the cuvette was 1 mm

FIG 8 Comparison of the spectra of reduced cytochrome *c* cooled to  $-72^{\circ}$  and  $-189^{\circ}$  The top curve represents the spectrum obtained when the sample of reduced cytochrome *c* was cooled in liquid air (Condition II), while the bottom curve is the spectrum of the same sample cooled to  $-72^{\circ}$  in an alcohol-dry ice mixture (Condition III) The sample cuvette contained a mixture of 0.1 ml of cytochrome *c* solution of  $2 \times 10^{-4}$  M, 0.4 ml of 0.1 M phosphate buffer, pH 7.4, a few crystals of sodium dithionite, and 0.5 ml of glycerol The reference cuvette contained a mixture of 50 per cent glycerol and 50 per cent 0.1 M phosphate buffer, pH 7.4 The effective band width was 0.6  $m\mu$ , the optical depth of the cuvette was 1 mm

in this manner, as well as the same sample after being treated in liquid air to Condition II The light absorption by the bands of the reduced hemoprotein cooled to  $-72^{\circ}$  is increased to about the same degree as with the treatment in liquid air However, the sharpening and splitting of

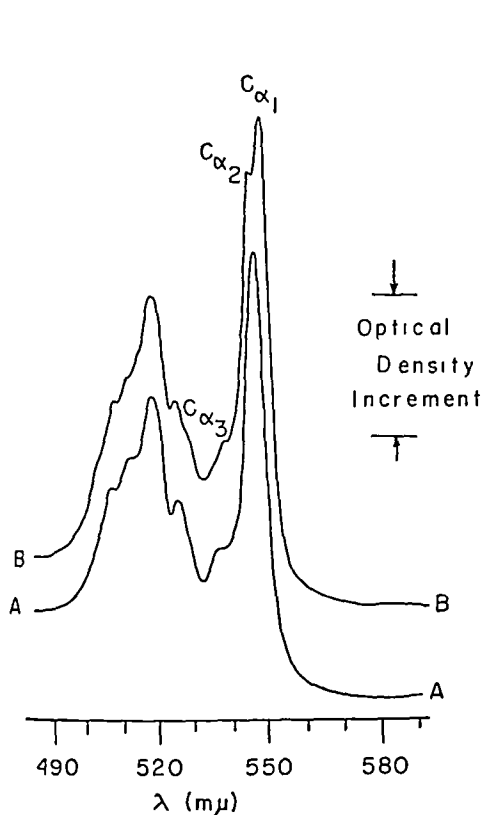


FIG 9

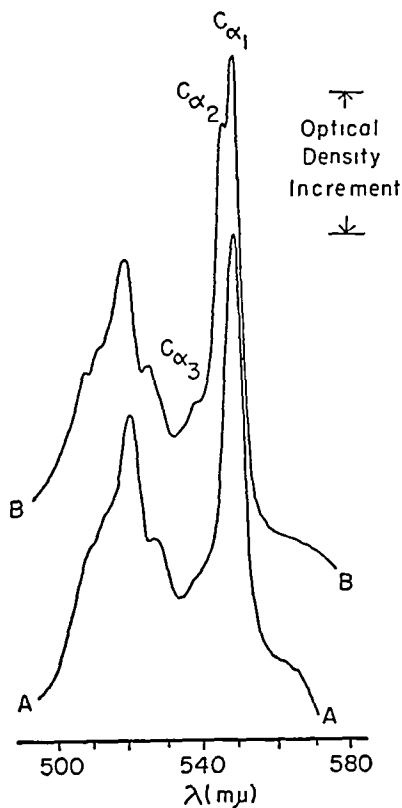


FIG 10

Fig 9 A comparison of the low temperature spectra of reduced cytochrome *c* prepared from yeast and heart muscle. Curve A, the low temperature spectrum of reduced cytochrome *c* prepared from yeast. The sample cuvette contained a mixture of 0.2 ml of yeast cytochrome *c*, 0.3 ml of 0.1 M phosphate buffer of pH 7.4, a few crystals of  $\text{Na}_2\text{S}_2\text{O}_4$ , and 0.5 ml of glycerol. Curve B, the low temperature spectrum of reduced cytochrome *c* from heart muscle. The sample cuvette contained a mixture of 0.1 ml of  $1.5 \times 10^{-4}$  M cytochrome *c*, 0.4 ml of 0.1 M phosphate buffer of pH 7.4, a few crystals of  $\text{Na}_2\text{S}_2\text{O}_4$ , and 0.5 ml of glycerol. The reference cuvette contained a mixture of equal volumes of glycerol and 0.1 M phosphate buffer of pH 7.4. The optical density increment was 0.10 for both samples. Optical depth of cuvettes, 1 mm, effective band width, 0.5  $\mu\text{m}$ , Condition II, temperature,  $-190^\circ$ .

Fig 10 A comparison of the low temperature spectra of reduced heart muscle cytochrome *c* and cytochrome *c*<sub>2</sub> from *R. rubrum*. Curve A, the sample cuvette contained a mixture of 0.4 ml of cytochrome *c*, 0.1 ml of 0.5 M phosphate buffer of pH 7.4, a few crystals of  $\text{Na}_2\text{S}_2\text{O}_4$ , and 0.5 ml of glycerol. Optical density increment, 0.04. Curve B, the sample cuvette contained 0.1 ml of  $1.6 \times 10^{-4}$  M heart muscle cytochrome *c*, 0.4 ml of 0.1 M phosphate buffer of pH 7.4, a few crystals of  $\text{Na}_2\text{S}_2\text{O}_4$ , and 0.5 ml of glycerol. Optical density increment, 0.10. The reference cuvette for both samples contained a mixture of equal volume of glycerol and 0.1 M phosphate buffer of pH 7.4. Optical depth of cuvettes 1 mm, effective band width, 0.6  $\mu\text{m}$ , Condition II, temperature,  $-190^\circ$ .



the bands are not pronounced at  $-72^{\circ}$ . This illustrates that the intensification of the absorption bands can be obtained at a relatively higher temperature than that needed to show the sharpening and fine structure of the bands.

*Cytochrome c from Other Sources*—Experiments were carried out to determine whether, when examined at low temperatures, similar pigments, prepared from yeast or *R. rubrum* showed the same type of fine line structure as did reduced cytochrome *c* of heart muscle.

TABLE I  
*Location of  $\alpha$ - and  $\beta$ -Band Maxima of Reduced Cytochrome c Cooled to Liquid Air Temperatures*

Band	Source of cytochrome		
	Heart muscle	Yeast	<i>R. rubrum</i>
$\alpha$	548.6	546.6	548.8
	545.9	536.6	538.4
	537.7		
$\beta$	528.5	526.2	527.3
	525.4	518.5	520.0
	518.5	512.3	513.8
	514.7	510.4	509.2
	511.6	507.0	
	507.8		
	503.1		

All wave length determinations are based on the maximum of the  $\alpha$ -band of reduced cytochrome *c* of heart muscle, which at room temperature is located at 550.0  $m\mu$  (12). The effective band width employed was 0.5 to 0.6  $m\mu$ .

Cytochrome *c*, when prepared from yeast, has a spectrum at room temperature which cannot be distinguished from heart muscle cytochrome *c*. When the reduced cytochrome *c* from yeast is treated in liquid air, the  $\alpha$ -band does not split (Fig. 9) in the manner described above for cytochrome *c* of heart muscle. The bands  $c_{\alpha_1}$  and  $c_{\alpha_2}$  are absent, and only an  $\alpha$ -band with a maximum at about 547  $m\mu$  can be observed. A band similar to  $c_{\alpha_3}$  and the multitude of  $\beta$ -bands are apparent in the yeast preparation. Similar spectra are obtained if the yeast cytochrome *c* is reduced chemically by sodium dithionite or enzymatically by yeast lactic dehydrogenase (cytochrome  $b_2$ ).

Cytochrome  $c_2$ , prepared from *R. rubrum*, when investigated at low temperatures showed a spectrum similar to but not identical with yeast cytochrome *c*. The spectrum of reduced cytochrome  $c_2$  (Fig. 10) shows the presence of an  $\alpha$ -band at 549  $m\mu$  apparently identical to the  $c_{\alpha_1}$  band

of reduced cytochrome *c* of heart muscle. The absence of any  $c_{\alpha_2}$  band, but the appearance of a band similar to  $c_{\alpha_1}$ , can be observed.

All three cytochromes described have a multitude of  $\beta$ -bands at low temperatures. The estimated locations of the  $\alpha$ - and  $\beta$ -bands of the three types of cytochromes are summarized in Table I.

The  $c_{\alpha_2}$  band at 546 m $\mu$  of cytochrome *c* of heart muscle is of greatest interest, for this band cannot be seen in the spectrum of the other types of cytochrome *c*. When the cytochromes of a heart muscle homogenate were reduced enzymatically by sodium succinate, bands similar to  $c_{\alpha_1}$  and  $c_{\alpha_2}$  were observed.<sup>3</sup> It therefore appears that the  $c_{\alpha_2}$  band is not due to an artifact which occurs during the rather drastic conditions necessary to prepare soluble cytochrome *c* of heart muscle.

These results indicate that the splitting of the  $\alpha$ -band of reduced cytochrome *c* of heart muscle into the two bands,  $c_{\alpha_1}$  and  $c_{\alpha_2}$ , may be due to two different pigments. Previous studies on the hemin of heart muscle cytochrome *c* (20) have shown that at least two hemins can be obtained from this pigment. This result, considered together with the data reported here, strengthens the supposition that preparations of "purified" heart muscle cytochrome *c* may consist of a mixture of hemoproteins. Further chemical studies on the hemins of the yeast and *R. rubrum* cytochrome *c*, as well as measurements on the comparative enzymatic activities of the pigments, will be necessary before this postulate can be confirmed.

Other work on the low temperature spectral properties of a variety of biological pigments will be reported in the near future. It is hoped that the above data presented for cytochrome *c* may aid in interpreting the biological function of this oxidation-reduction catalyst.

The author is grateful to Dr. Britton Chance and his colleagues for advice and assistance during the course of this work. The cooperation of Mr. Patrick Taylor in measuring the changes in temperature and Mr. V. Legallais for the design and construction of the adapter used with the spectrophotometer is appreciated.

#### SUMMARY

A method is described for recording spectrophotometrically the absorption bands of samples of hemoproteins cooled in liquid air. The application of the technique to a study of the spectral properties of reduced cytochrome *c* from heart muscle, yeast, and *Rhodospirillum rubrum* has been summarized. The results demonstrate the sharpening, splitting, and intensification of some of the absorption bands of the reduced hemoproteins. The usefulness of the method for recognizing spectral differences of hemoproteins, which at room temperature have similar absorption bands, is shown by a com-

parison of the  $\alpha$ - and  $\beta$ -bands of the reduced cytochromes prepared from the three different sources

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# STUDIES OF METABOLIC TURNOVER WITH TRITIUM AS A TRACER

## V THE PREDOMINANTLY NON-DYNAMIC STATE OF BODY CONSTITUENTS IN THE RAT\*

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The pioneering isotopic tracer studies of Schoenheimer and coworkers, in which deuterium- and  $N^{15}$ -labeled compounds were utilized in the measurement of metabolic turnover rates, have been widely accepted as convincing evidence for the essentially dynamic state of most, if not all, body constituents (1). The "dynamic state of body constituents" has become a byword in most modern textbooks of biochemistry, and Folin's earlier distinction between endogenous and exogenous metabolism of proteins (2) has been usually treated as a historic, but outgrown concept. In recent years it has become evident, however, that not all body constituents are so very dynamic. These findings have been recently reviewed by Mitchell (3), who finds them in no sense incompatible with the essential features of Folin's original concept. Despite this more recent evidence for non-dynamic components in many tissues, the results of short term experiments of isotope retention are still frequently interpreted as indicating rapid turnover rates for the total amount of the constituent under investigation. Such interpretations ignore the fact that the presence of a long lived fraction could not possibly be detected during the short experimental periods employed.

In experiments similar to the early deuterium work of Schoenheimer *et al* (1), but with tritium as a tracer, we have previously demonstrated the existence in the rat of metabolically inert components with apparent biological half lives of the order of 100 days or longer (4, 5). These experiments involved the administration of a single large dose, or a short series of doses, of tritium oxide. The amount of tritium incorporated in the long lived components was always small compared to that incorporated in the more dynamic components. The long lived components were discernible only by virtue of the wide range of concentration over which tritium may be detected, which allowed us to trace the tritium content of

\* This paper is based on work performed under contract No W-31-109-Eng-52 for the Atomic Energy Commission

the animals until the shorter lived components were eliminated. While these experiments clearly demonstrated the existence of metabolically inert components in nearly all tissues, only rough estimates of pool sizes were possible.

We have now completed chronic exposure studies which are susceptible to a more quantitative interpretation. These studies bear out the earlier indications that the concept of the dynamic state of body constituents is more restricted in its applicability than has generally been supposed.

### Methods

Twelve female rats of the Sprague-Dawley strain, average weight 235 gm, were injected intraperitoneally with sufficient tritium oxide to bring the concentration of tritium in their body water to approximately 34  $\mu\text{c}$  per ml. Thereafter, for a period of 124 days, all drinking water available to these animals contained 50  $\mu\text{c}$  per ml of tritium as the oxide. Purina laboratory chow was available *ad libitum* throughout the experiment. After 6 weeks on the tritium oxide regimen, the animals were mated. The offspring were exposed to the uniform maternal tritium oxide environment *in utero*, were nursed by the mothers treated with tritium, and weaned to the regimen of tritium oxide-labeled drinking water, which was maintained until the animals were 6 months old.

After cessation of tritium oxide administration, groups of rats from both generations were killed at intervals as indicated in Tables I and II, and the organically bound tritium content of various organs and tissues was determined. Samples were pooled from the two or three animals in each of these groups. Total samples were taken except in the case of fat (genital and perirenal), pelt (dorsal), bone (femora), and muscle (hind leg). The residual carcass was thoroughly ground and the aliquots were analyzed. The methods employed in the removal of water from the tissues and in the combustion of the dry residue have been previously described (6). Samples were not equilibrated with water (7), therefore, the values for organically bound tritium include "freely exchangeable" as well as "firmly bound" tritium.

In addition to the samples of organ and tissue, organically bound tritium was determined in fractions separated from the residual carcass. The fractions studied are listed in Tables I and II, the methods employed in their separation having been previously described (5). The fraction listed as "insoluble residue" consists of material left after removal of water soluble, fat-soluble, and NaOH-soluble fractions.

The tritium-counting procedure has been previously described (7, 8).

## Results

Experimental results are summarized in Table I (first generation rats) and Table II (second generation rats) All the data are calculated in

TABLE I  
Concentration of Tritium in Organically Bound Hydrogen of Rats after  
Chronic Exposure to Tritium Oxide for 4 Months during Maturity

Group No * Time killed, days Average rat weight, gm		1 0 249	2 10 245	3 30 259	4 93 266	5 184 258	6 360 300
Tritium concentration in water from combustion as per cent of concentration in body water during exposure†							
Tissues	Carcass	21	13	8 4	4 4	2 8	0 81
	Liver	28	8 9	2 2	0 57	0 29	0 086
	Lung	16	12	3 1	0 97	1 03	0 32
	Heart	9 5	11	5 3	1 03	1 03	0 25
	Kidney	25	11	3 5	1 2	0 65	0 32
	Stomach	18	9 7	4 7	3 1	2 3	0 84
	Small intestine	20	8 7	3 8	1 5	1 00	0 33
	Large "	15	8 4	5 7	2 8	1 9	0 81
	Brain	22	18	12	6 2	3 8	1 4
	Pelt	24	14	12	7 6	4 0	1 7
	Muscle	23	14	9 2	4 8	3 0	0 46
	Fat	15	12	9 2	4 0	3 6	0 30
	Bone	18	4 6	3 2	3 2	3 0	1 8
	Phospholipides	17	12	9 2	6 0	4 0	0 62
	Non-saponifiable lipides	17	15	7 3	5 4	2 4	1 4
Compound fractions	Saturated fatty acids	15	12	8 6	5 7	7 0	1 4
	Unsaturated fatty acids	7 0	7 0	4 6	6 0	8 8	2 5
	Collagen	1 9	3 2	3 5	3 4	3 4	2 8
	Water-soluble	15	13	7 0	3 4	3 4	0 24
	Alcohol-ether-insoluble	17	13	8 9	2 2	0 73	0 35
	Insoluble residue	9 7		4 8		2 8	1 2

\* Each group consisted of two animals, samples from which were pooled prior to analysis

† Average concentration of tritium in body water during exposure was 3.7  $\mu$ c per ml. The values were normalized as explained in the text

terms of the tritium concentration in water derived from the combustion of the dry tissue, or compound fraction, and expressed as a per cent of the concentration of tritium which was maintained in the body water throughout the exposure period. By expressing tissue-bound tritium in terms of tritium concentration in the water derived from the combustion of the tissue, all figures become proportional to the specific activity of tritium in

hydrogen, thus simplifying comparisons among compounds of various hydrogen contents and body water. The average concentration of tritium in body water maintained during the period of chronic exposure was 3.7

TABLE II

*Concentration of Tritium in Organically Bound Hydrogen of Rats after Chronic Exposure to Tritium Oxide from Conception to 6 Months of Age*

Group No	1	2	3	4	5	6	7	8	9
Sex	M	F	F	F	M	F	M	M	M
No. of rats	3	3	3	3	3	3	3	2	2
Time killed, days	0	0	5	14	35	70	119	200	300
Average rat weight, gm	368	217	247	246	390	221	396	508	399

Tritium concentration in water from combustion as per cent of concentration in body water during exposure\*

Tissues	Carcass	19	17	18	15	10.5	5.4	4.6	4.0	1.8
	Liver	27	27	20	8.1	2.3	0.62	0.45	0.48	0.19
	Lung	24	26	16	7.6	5.9	2.9	2.7	2.5	1.0
	Heart	21	22	19	12	6.5	2.1	1.1	3.8	1.5
	Kidney	26	25	20	9.5	3.9	1.5	1.2		0.09
	Stomach	24	26	20	12	8.3	3.2	3.8	3.9	2.5
	Small intestine	21	21	19	9.7	3.9	2.6	1.7	1.6	0.97
	Large "	20	22	20	12	6.5	5.3	2.9	3.1	2.1
	Brain	37	39	32	30	11	13	13	10	5.1
	Pelt	28	22	25	21	17	12	8.0	6.2	2.7
	Muscle	23	18	20	16	11	5.8	4.2	3.2	1.3
	Fat	12	11	15	13	8.1	4.6	3.4	1.9	0.44
	Bone	17	16	18	14	11	9.5	8.1	7.6	4.9
Compound fractions	Phospholipides	21	17	20	17	10.3	7.6	4.6	4.1	3.0
	Non-saponifiable lipides	26	20	15	17	10.3	10.6	6.0	4.3	3.8
	Saturated fatty acids	11	10.3	13	13	7.8	4.9	3.2	1.7	0.46
	Unsaturated fatty acids	7.8	5.9	8.4	7.3	4.1	3.4	2.1	1.1	0.46
	Collagen	22	20	20	17	16	15	12	16	12
	Water-soluble	16	18	18	12	6.8	4.0	2.4	0.62	0.32
	Alcohol-ether-insoluble	25	24	20	17	13	8.6	6.2	4.4	3.6
	Insoluble residue	14	14	12	9.5		4.0	5.4	3.4	3.1

The values were normalized as explained in the text

\* The average concentration of tritium in body water during exposure was 3.7  $\mu\text{C}$  per ml

$\mu\text{C}$  per ml The percentage values listed in Tables I and II may therefore be converted to microcuries per ml by multiplying by 0.037

The decrease in tritium concentration of tissue after exposure may result not only from metabolic processes, but also as a consequence of simple dilution due to growth of the animal. In an effort to eliminate this effect of dilution, data in Tables I and II, where necessary, have been normalized to a constant weight of organ, tissue, or compound fraction. For

those organs analyzed *in toto*, the standard taken for normalization was the average organ weight in the first group of the second generation rats which were killed. Where only partial tissue samples were taken, normalization was based on total animal weight, with the first group of second generation rats being taken as the standard. Data for male and female animals were separately normalized. With the exception of Group 6 in Table I and Group 8 in Table II, weight differences at death were relatively slight, and the normalization procedure introduced only small changes in tritium concentrations.

Data from the separated compound fractions, with one exception, were not normalized. It was felt that the small differences in "fraction yield" among the groups of rats were just as attributable to chemical and manipulative losses as to differences due to animal growth. The single exception was the case of saturated fatty acids which, in the first generation animals, increased quite significantly in Groups 5 and 6.

#### DISCUSSION

*Radiation Dose*—In previous studies in this series, it was sometimes necessary to employ concentrations of tritium which irradiated the rats at levels as high as 30 rads per day for periods of several days (5). While no gross symptoms of radiation damage were observed, such high levels of radiation are undesirable because of their possible effects on the general metabolism of the animal. In the present study, the continuous level of  $3.7 \mu\text{c}$  of tritium per ml of body water gave a maximal dose rate to the animal of only 1 rad per day.

*Metabolic Incorporation of Hydrogen from Body Water*—Organically bound hydrogen in an animal may arise from two sources. (a) It may be present, already bound, in organic molecules taken in as food, these molecules or portions thereof being incorporated into the tissue compounds of the animal. (b) It may be incorporated from the hydrogen of body water during the metabolic synthesis of tissue compounds. In the present study the second generation rats killed at the end of the exposure period (Groups 1 and 2, Table II) were exposed throughout their existence to body water hydrogen containing a constant proportion of tritium. The organically bound hydrogen in their food contained no tritium. The tritium content of the tissue compounds of these animals should therefore indicate the extent to which hydrogen is derived from each of the two available sources, for the moment the possibility of isotopic differentiation being excluded. The results for Groups 1 and 2 (Table II) indicate that, for most of the samples analyzed, from 20 to 30 per cent of the organically bound hydrogen was apparently derived from body water. Several of these samples were well outside this range, however, and require additional comment. Brain



tissue was uniquely high in bound tritium, with results which indicate that nearly 40 per cent of its hydrogen was derived from body water. This leads to the conclusion that brain, to a greater extent than other tissue, is composed of materials synthesized within the body. Such a finding may be related to the existence of the so called "blood-brain barrier" which might be expected to hinder incorporation of large preformed molecules into the brain substance (9, 10). Thus Friedberg and Greenberg have observed an apparent barrier to the uptake of amino acids by the brain (11). The fact that the composition of brain lipides, as compared with other organ lipides, is least influenced by diet (12) is also in qualitative accord with this finding.

The separated fatty acid fractions and the gross fat sample were notably low in bound tritium, indicating that these materials, to a greater extent than other tissue components, were derived from preformed organic molecules taken in as food. This conclusion is consistent with many studies which have demonstrated the extensive direct incorporation of fatty acids from food into depot fats (13). The lesser incorporation of tritium into the unsaturated as compared to the saturated fatty acids corresponds to previous findings with deuterium (14).

The tritium concentration in certain tissues from the first generation animals (Table I, Group 1) is significantly lower than in corresponding tissues from second generation animals (Table II, Groups 1 and 2), indicating that the 4 months exposure of the first generation rats was insufficient to permit equilibrium levels of tritium to be attained in some of the more metabolically inert fractions. Most notable is the case of collagen, in which tritium incorporation is 10-fold higher in the animals exposed from conception. It seems quite evident that the bulk of the collagen in the first generation rats had been formed prior to the tritium oxide exposure and that little degradation and resynthesis of this collagen occurred during the 4 month exposure period. The metabolic inertia of collagen was indicated in previous studies by ourselves (5) and others (15-17) and is borne out by other features of the present investigation, to be discussed subsequently.

In the foregoing discussion no account has been taken of the possibility that the incorporation into tissue compounds of tritium from body water may not be a true measure of hydrogen incorporation. It is evident from recent studies by ourselves (7) and others (18, 19) that differences do exist in the rates of incorporation of deuterium and tritium under circumstances similar to those of the present experiment. Ratios of tritium to deuterium incorporation as low as 0.77 have been reported for the case of mammary gland fatty acids (18). It is likely that even larger differences may exist between tritium and protium (ordinary hydrogen) incorpora-

tion It is therefore reasonable to suppose that the incorporation into tissue compounds of hydrogen from body water in the rats of the present experiment was actually somewhat higher than indicated by the results with tritium While this uncertainty would preclude any inferences based on absolute values of tritium incorporation, it is not felt that it significantly affects any of the comparisons considered in the preceding discussion

It should also be pointed out that the fraction of organically bound hydrogen which is derived from body water is undoubtedly a function of diet Thus Bernhard and Schoenheimer found that, on a fat-free diet, approximately 50 per cent of hydrogen in saturated fatty acids was derived from body water, as indicated by deuterium incorporation (14) This is in contrast to our result of 10 to 15 per cent as indicated by

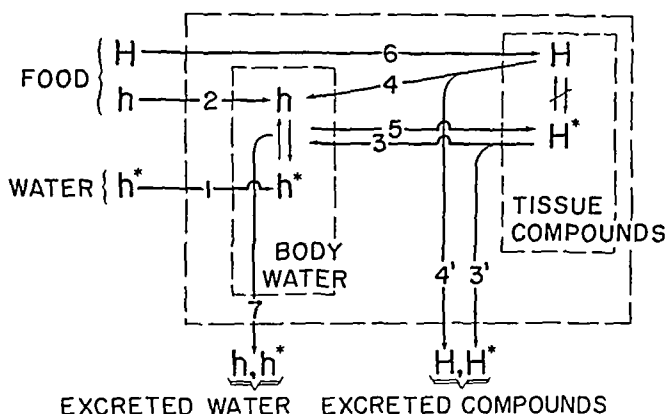


Fig 1 Simplified scheme of hydrogen metabolism with ingested water labeled with tritium H, organically bound hydrogen, h, water hydrogen, \*, tritium label

tritium incorporation, and with a diet containing not less than 5 per cent crude fat The difference in these two results is probably owing largely to differences in the fat content of the diets rather than to an isotope effect

*Metabolic Inertia of Tissue Components As Measured by Tritium Retention*—The principal purpose of the present experiment was to obtain data from which the size of the metabolically inert hydrogen pools could be estimated A schematic representation of the metabolic systems involved is shown in Fig 1 Tritium-labeled hydrogen, from drinking water, enters the body water pool by Route 1 Non-labeled hydrogen from water, from food, enters by Route 2 Hydrogen also enters the pool of body water from the catabolism of tissue compounds, Route 3 representing the contribution from tritium-labeled compounds and Route 4 the contribution from unlabeled compounds The pool of body water is not separated into compartments, all portions having the same concentration of tritium, which is accurately reflected by any sample of body water

The organically bound hydrogen of tissue compounds is not uniformly labeled with tritium. Bound hydrogen arising from body water via Route 5 will be labeled to the same extent as hydrogen of body water, while bound hydrogen incorporated directly from food (Route 6) will be unlabeled. The same compound, of course, may, and probably in most cases will, contain both hydrogen derived from body water and hydrogen derived from food. The organically bound hydrogen pool consists of a great many compartments, the hydrogens of which will usually not equilibrate with each other.

Because of much justifiable criticism of the non-rigorous methods employed in the interpretation of tracer studies in biological systems (*e.g.*, Reimer (20)), it seems desirable at this point to define clearly the recognized limitations of the present study. The biological systems involved encompass the total animal. The complexity and lack of understanding of these systems are such that no attempt at a rigorous mathematical interpretation of the results is considered justified. Our aim is nothing more than a gross demonstration of the relative metabolic inertia of a great variety of tissue components and a semiquantitative indication of the magnitude of these relatively inert components. In performing this demonstration we will consider gross retention curves as being the sum of exponential components, and derive half lives for these components. In so doing, it is recognized that these exponential components and their associated half lives bear no clearly definable relationship to any specific biological component. These exponential components do, however, afford a fairly accurate representation of the net rate at which tritium is being lost from the tissue under investigation, and this loss of tritium can be, at least semiquantitatively, related to the rate of degradation of the compounds which compose the tissue.

In Fig. 2, data are presented on the retention of tritium in the second generation rats after removal of tritium from the drinking water. The organically bound tritium curve is derived from analyses on the "residual carcass," but may be considered as representative of the total animal. Within 10 days the concentration of tritium in hydrogen of body water has dropped to about the same level as tritium in organically bound hydrogen, and by 25 days the tritium concentration in body water hydrogen is less than one-tenth of that in organically bound hydrogen. Because of this rapid decrease in body water tritium, there is no possibility of a continued significant recycling of tritium between body water and tissue compounds. Upon removal of tritium from Route 1 (Fig. 1), tritium incorporation via Route 5 rapidly dwindles to insignificance, and the loss of organically bound tritium from the tissue compounds becomes a measure of the breakdown or excretion of these compounds via Routes 3 and 3'.

Although this measure of tissue degradation applies in a strict sense only to those components whose hydrogen was derived from body water, it can be assumed that it reasonably represents the total tissue. In other words, it can be assumed that Routes 3 and 3' are equivalent to Routes 4 and 4'. There would seem to be no *a priori* reason for assuming that the origin of a compound's hydrogen from food molecules or from body water should play a determining role in the subsequent fate of that compound, particularly since most compounds will contain hydrogen from both sources.

By equating loss of tritium via Routes 3 and 3' with the breakdown or

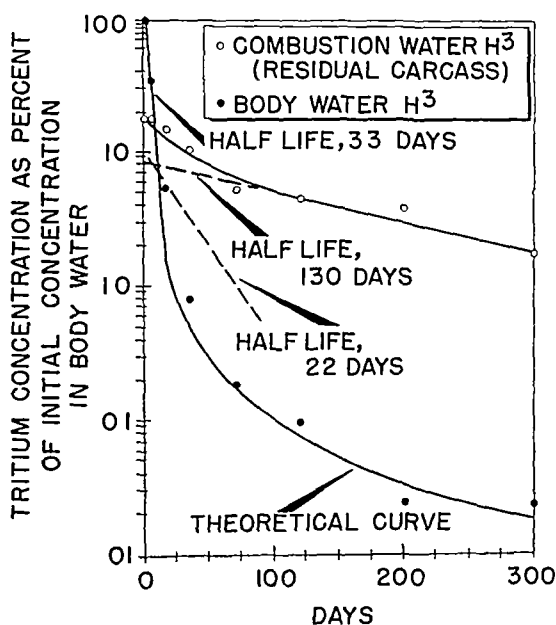


Fig 2 Retention of body water tritium and organically bound tritium in rats after chronic exposure to tritium oxide from conception to 6 months of age

excretion of labeled compounds, one assumes that the tritium is not lost by simple exchange with protium from body water. Tritium in labile positions, *e.g.* attached to oxygen or nitrogen atoms, is certainly rapidly lost by such an exchange process. The proportion of such labile hydrogen in the body is small, however, and much careful work with deuterium has shown that, with few exceptions, hydrogen linked to carbon is not exchanged under physiological conditions (1). The possibility of a very slow rate of exchange of carbon-linked tritium with body water protium, however, cannot be ruled out. If such an exchange contributes appreciably to the loss of tritium from tissue compounds, the neglect of this factor in our calculations will result in an *underestimation* of the biological half-lives of the compound turnover processes.

In the case of the second generation rats, we shall assume that the amount of tritium present in any gross organic tissue component at the conclusion of the tritium oxide feeding period is proportional to the mass of that component. Thus, if half the tritium in a given tissue is lost with a half life of 100 days, we shall assume that half of the tissue is being degraded and resynthesized with this half life. This amounts to an assumption that all organic tissue constituents contained the same proportion of uniformly labeled hydrogen at the conclusion of the exposure period. It was hoped that the long uniform exposure from conception to 6 months of age would result in reasonably uniform tritium labeling. As previously mentioned (Table II, Groups 1 and 2), complete uniformity among tissues and compound fractions was not achieved. It is felt, however, that the assumption of uniform labeling will introduce no greater error than that inherent in other factors in the interpretation.

It should be remarked that the possibility of isotopic differentiation between tritium and protium is of no significant concern in the interpretation of tritium retention. Once incorporated in the organic molecule, the release of tritium will be largely determined by the metabolic fate of the entire molecule, which will not be significantly influenced by a mass difference of 2 units in the total molecular weight. This conclusion was borne out in experiments in which the retention of organically bound tritium and deuterium in rats was compared (7).

In accord with the above considerations, we may now return to the interpretation of the organically bound tritium retention curve of Fig 2. This curve may be resolved, as shown, into two exponential components with half lives of 130 and 22 days. Extrapolation of these components to zero time indicates their relative magnitude, the 130 day component accounting for nearly half of the total tissue. We shall emphasize again that such resolution of a retention curve does not imply that two discrete biological components with these half lives exist. Rather, one should conclude that the many components which must exist in the animal may be grouped in this manner and that the "average half life" of the more inert group of components is 130 days, while that of the more dynamic group of components is 22 days. Such a grouping does not preclude the acknowledged existence of components with very short half lives. It does, however, indicate that such short half life components are quantitatively insignificant in the over-all picture for the total animal.

The biological half life of tritium in the body water of the rat, as deduced from body water analyses on the early groups of the animals killed, was 33 days, the same value as that determined in previous acute exposure studies (4). By knowing the rate at which tritium is lost from body water and the rate at which tritium is being introduced into the body water by

breakdown of tissue compounds (with an assumption that all released tritium follows Route 3 (Fig 1)), one can calculate the concentration of tritium which should be present in the body water throughout the period following cessation of tritium oxide feeding. Such a calculation results in the curve drawn for body water tritium in Fig 2. The comparison of this curve with the experimentally determined values serves as an excellent internal check on the general validity of the experimental and interpretative procedures.

In a manner similar to that illustrated for the residual carcass in Fig 2,

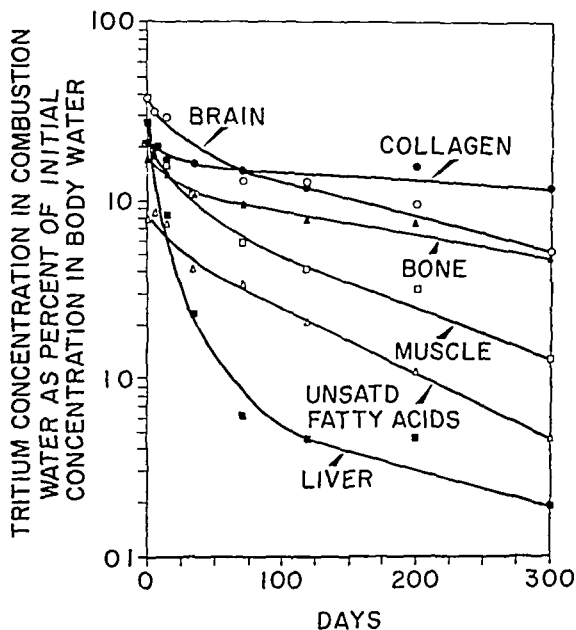


Fig 3 Retention of organically bound tritium in rat tissues and compound fractions after chronic exposure to tritium oxide from conception to 6 months of age

retention curves were plotted for all organs, tissues, and separated compound fractions by using the data from Tables I and II. Fig 3 shows several such curves for representative samples. These retention curves were resolved into exponential components and the half lives and relative magnitudes of the components determined. Table III summarizes the results from the second generation animals, the data from the first generation rats having led to similar results in most cases. The results from the first generation animals are not shown, since the assumption of uniform labeling involved in the evaluation of the magnitude of the components is more questionable for these rats.

It was possible to resolve components with half lives as short as 3 to 5 days for only four organs. This does not mean that such dynamic com-

ponents were not present in the other samples, but simply indicates that, owing to the predominance of longer lived components, it was impossible to resolve components of very short half life accurately

Relatively inert components with half lives, in most cases longer than 100 days, constitute at least 30 per cent of all the samples analyzed, except liver, kidney, lung, stomach, and small intestine Heart might also be

TABLE III  
*Magnitude and Biological Half Life of Tissue Components of Rat*

		Long lived component		Shorter lived components
		Magnitude, per cent total tissue or fraction	Biological half life	Biological half life
			days	days
Tissue	Carcass	47	130	22
	Liver	3	140	12, 4 5
	Lung	14	320	10, 3
	Heart			
	Kidney	8	180	11
	Stomach	20	300	20, 5
	Small intestine	17	160	9
	Large "	30	180	13, 5
	Brain	54	150	16
	Pelt	67	110	11
	Muscle	40	100	16
	Fat	69	70	17
	Bone	72	240	16
Compound fractions	Phospholipides	33	220	20
	Non-saponifiable lipides	50	160	20
	Saturated fatty acids	60	80	15
	Unsaturated fatty acids	74	80	10
	Collagen	72	1000	15
	Water-soluble	50	60	10
	Alcohol-ether-insoluble	36	200	25
	Insoluble residue	40	300	15

expected to fall in this category, but the data obtained for heart were too erratic for accurate resolution of the retention curve Even these metabolically active organs possess well defined components with half lives exceeding 100 days

The greatest degree of metabolic inertia is exhibited by collagen, with 72 per cent of the fraction exhibiting an apparent half life of 1000 days From the scatter of points on the collagen retention curve (Fig 3), it should be evident that the half life could have been assigned any value from 500

days to infinity about as reasonably. The conclusion would seem to be justified that most of the collagen of mature rats is not replaced during the lifetime of the animal. This conclusion is supported by the 10-fold lower incorporation of tritium in the first generation animals which were exposed after attaining essentially full growth.

While the existence of metabolically inert collagen (15-17), and protein fractions generally (21-24), has been demonstrated by other investigators, the extreme metabolic inertia of a substantial fraction of body lipides seems to have been first indicated by earlier studies in this series (4, 5). Other workers have determined biological half lives for various lipid components of the rat, using isotopic tracer methods, with resulting values which range from 1 to 35 days (25-27). In all cases, these values were based on observations extending over short time periods (not exceeding 30 days), and the assumption was made that the half life observed applied to the total lipid fraction under investigation. It should be evident from the present study that these short half lives are not representative of the total lipid fractions, but apply only to the more dynamic portions of these fractions. The present data indicate that relatively inert components constitute the majority of body lipides generally, and approximately two-thirds of both saturated and unsaturated fatty acids.

A recent report by Steele (28) on the retention of  $C^{14}$  in tissues of the mouse after a single ingestion of uniformly labeled sucrose furnishes support for our hypothesis of the prevalence of metabolically inert components in most tissues. Although extending over a period of only 36 days, his data indicate components with apparent half lives of from about 17 to 33 days in all the tissues examined. His value of 33 days for mouse muscle compares with our finding in a 31 day experiment of an apparent half life of approximately 30 days for the organically bound tritium of the total mouse (6). Thus the results with hydrogen labeling are in excellent agreement with the results from a comparable experiment in which the carbon was labeled directly. In comparing these two studies, it becomes evident that the use of tritium as a label offers at least two advantages over the apparently more straightforward carbon label. First, the uniform labeling of tissue compounds is certainly more closely approached with chronic tritium oxide administration than with the feeding of any carbon-labeled substance, and second, the greater probability of carbon reutilization, as compared to hydrogen reutilization, makes quantitative interpretation of  $C^{14}$  retention data much more difficult.

#### SUMMARY

Rats exposed to a constant level of tritium oxide in body water from conception to 6 months of age were subsequently killed at time intervals ex-



tending to 300 days. Another series of animals, similarly exposed for a period of 124 days after attaining maturity, was subsequently killed at intervals extending to 360 days. Organically bound tritium was determined in various organs, tissues, and compound fractions, and the results were interpreted as follows:

1 Under the dietary conditions employed, without consideration for the possible effects of isotopic differentiation, from 20 to 30 per cent of the hydrogen of most tissue compounds was derived from body water. Brain and fatty acids were exceptional, with nearly 40 per cent of brain hydrogen and about 10 per cent of fatty acid hydrogen derived from body water.

2 Dynamic components (half lives of a few days) constitute a very small proportion of the total animal, and in the present experiment were distinguishable from the predominant "less dynamic" components only in liver, lung, stomach, and intestine.

3 Approximately half of the organic materials constituting the total rat is being degraded and resynthesized with apparent biological half lives longer than 100 days.

4 Collagen was found to be uniquely inert. Most of the collagen of mature rats is apparently not replaced during the lifetime of the animal.

5 The majority of body lipides, including about two-thirds of both saturated and unsaturated acids, exhibit half lives of the order of 70 days or longer.

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# THE BIOSYNTHESIS OF MONOMETHYLETHANOLAMINE BY NEUROSPORA CRASSA\*

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The formation of the methyl groups of choline and methionine from  $C^{14}$ -labeled formate has been demonstrated in rat liver slices (1) and in the intact rat (1-5). The administration of sodium deuterio- $C^{14}$ -formate in the rat was followed by the isolation of tissue choline containing  $C^{14}$ -labeled methyl with no detectable loss of deuterium (6). Stekol *et al* (7) concluded that choline is synthesized in the rat by the direct transfer of a methyl group from methionine to dimethylethanolamine and that methionine may not participate directly in the formation of dimethylethanolamine. Their data indicate also that the *de novo* synthesis of the two methyl groups of dimethylethanolamine is mediated by a folic acid derivative and that folic acid and its derivative are not involved in the transfer of the methyl group of methionine to dimethylethanolamine. These investigations have utilized the isolation and analysis of labeled choline or betaine and not of the intermediate mono- and dimethylethanolamines. Additional evidence is required therefore to permit an unequivocal differentiation between steps in the synthesis of choline which may involve methylation by conversion of a formate to a methyl carbon as well as the direct transmethylation of methyl from methionine.

In the present paper sodium formate- $C^{14}$  and L-methionine- $CH_3^{14}$  (Isotopes Specialties Company, Inc.) have been compared as sources of the methyl of the monomethylethanolamine synthesized by mutant strains of *Neurospora crassa*.

## EXPERIMENTAL

*Neurospora Mutants Employed*—*Neurospora* strain 47904, previously described by Holowitz *et al* (8, 9), synthesizes monomethylethanolamine but, because of a mutation of a genetic locus concerned with the synthesis of choline, is unable to convert the monomethylethanolamine to choline at the normal rate. Consequently, the intermediate accumulates in the culture. Strain C-24, first isolated by Mrs. Mary B. Mitchell (10) and later described by Harold and Fling (11), requires exogenous formate for growth.

\* This investigation was supported by a research grant from the National Vitamin Foundation.

Its nutritional deficiency is presumably due to a mutation concerned with the normal production of formate from serine. A third *Neurospora* mutant employed in these studies, strain 38706, also described by Horowitz (12), is dependent for growth on exogenous methionine, since it is unable to methylate homocysteine. The above organisms were put through the appropriate genetic crosses to produce double mutant strains 47904  $\times$  C-24 and 47904  $\times$  38706. The identity of each double mutant was verified by outcrossing with the standard strain. Both organisms require choline for growth and accumulate monomethylethanolamine. In addition, strain 47904  $\times$  C-24 requires exogenous formate and strain 47904  $\times$  38706 requires exogenous methionine. Strains 47904  $\times$  C-24 and 47904  $\times$  38706 were grown under identical conditions on media containing formate- $C^{14}$  and L-methionine- $CH_3-C^{14}$ , respectively. The incorporation of isotope into the methyl moiety of the accumulated monomethylethanolamine was compared in the two organisms.

*Growth of Microorganisms*—In each experiment a 2 liter culture of either *Neurospora* strain 47904  $\times$  C-24 or 47904  $\times$  38706 was grown for 10 days at 25° under forced aeration on a minimal medium (13) containing 232  $\mu$  of choline chloride per culture. In addition to the choline supplement the 47904  $\times$  C-24 culture medium contained 150 mg of sodium formate- $C^{14}$  and the 47904  $\times$  38706 medium contained 40 mg of L-methionine  $CH_3-C^{14}$ . Each supplement was used at the minimal concentration level required for optimal growth of the molds. Although the double mutants exhibited no growth on minimal medium, growth on the supplemented media approached that of the normal strain and, accordingly, the nutritional requirements of both organisms were satisfied by the supplemented media.

*Isolation and Degradation of Monomethylethanolamine from Neurospora*—Concentrates of monomethylethanolamine were obtained from the mold by essentially the procedure originally described by Horowitz (9). The only step that was omitted was the removal of choline as the reneckate, since this separation was accomplished later in the isolation procedure. The monomethylethanolamine was purified on a 15  $\times$  265 mm chromatographic column with Dowex 50 cation exchange resin in the hydrogen form as the stationary phase and 1.5 N HCl as the eluting solvent. At a flow rate of about 14 ml per hour, the methylethanolamine emerged from the column after approximately 190 ml of eluate had been collected, and the bulk of the substance was contained in about 30 ml of the following eluate. Preliminary experiments with authentic samples showed that this column separates monomethylethanolamine from dimethylethanolamine and choline quantitatively. The separation is essential for the successful quantitative bioassay of these materials with *Neurospora* mutants (14).

The amount of monomethylethanolamine isolated by this procedure

from a 2 liter culture of either one of the double mutants was about 1 mg. The exact amount was determined by assay with *Neurospora* strain 34486 (14), and at this point in the procedure the material was diluted about 200-fold with carrier. The diluted amine was extracted from an aqueous alkaline solution with butanol and was recrystallized to constant isotope activity from ethyl acetate as the picric acid salt (15). Monomethylethanolamine treated with a stoichiometric amount of periodic acid at room temperature yields 1 mole of methylamine and 2 moles of formaldehyde<sup>1</sup>. This method was employed to degrade the amine in order to determine the distribution of isotope activity in the three carbons. The methylamine obtained by this procedure was purified as a chloroplatinate and the formaldehyde as the dimethone derivative.

*Tracer Studies*—The monomethylethanolamine picrate and methylamine chloroplatinate were oxidized to CO<sub>2</sub> and counted in a flow counter as BaCO<sub>3</sub>. The formaldehyde was plated as the dimethone derivative. The appropriate corrections were made for self-absorption and backscattering.

#### DISCUSSION

Tables I and II present the data on three experiments with strain 47904 × C-24 and two experiments with strain 47904 × 38706. Originally only two duplicate experiments for each strain were planned, but, in view of the unexpected high activity found in the methyl moiety of monomethylethanolamine from strain 47904 × C-24, a third experiment was performed at a later date as a precautionary measure.

Expressed in terms of atomic or molar ratios, the data show that, when the carbon in a mole of supplemental formate is incorporated in the methyl group of monomethylethanolamine, it is diluted in the process by only 0.5 gm atomic weight of carbon coming from endogenous sources. In contrast to this, the carbon in the methyl group of supplemental methionine is diluted about 7-fold before it is incorporated into the methyl of the same methylamine. This high incorporation of activity from formate to the methyl group of monomethylethanolamine is evidence that formate is a fairly direct precursor of this moiety in *Neurospora*. The much lower transfer of activity from the methyl group of methionine to the methyl of monomethylethanolamine indicates a less direct metabolic pathway for this methyl to methyl transfer. These results are in keeping with the findings of Stekol *et al* (7) that the *de novo* synthesis of the two methyl groups of dimethylethanolamine is mediated by a folic acid derivative in the rat. Whereas the involvement of folic acid derivatives in formate metabolism is recognized, existing evidence does not indicate that folic acid or its biological derivative is a cofactor in the enzyme reactions involv-

<sup>1</sup> Unpublished work

ing transmethylation from methionine (7) The role of folic acid in the biosynthesis of choline by *Neurospora* is under investigation in this laboratory

It is necessary to distinguish carefully between methylation due to trans methylation and methylation due to synthesis of a methyl from formate Particularly disturbing in the past has been the difficulty in describing the

TABLE I  
*Distribution of C<sup>14</sup> in Monomethylethanolamine from  
Neurospora, Strain 47904 × C-24*

Experiment No	N-Methyl carbon		Carbons 1 and 2 of ethanolamine 2 carbon residue	
	Specific activity, c p m per mg carbon × 10 <sup>-5</sup>	Per cent of original specific activity in formate*	Specific activity, c p m per mg carbon × 10 <sup>-5</sup>	Per cent of original specific activity in formate
I	8 1	69 2	4 1	0 35
II	7 8	66 6	3 9	0 33
III	7 7	65 8	5 3	0 45

\* The specific activity of the administered sodium formate-C<sup>14</sup> was  $1.17 \times 10^5$  c p m per mg of carbon

TABLE II  
*Distribution of C<sup>14</sup> in Monomethylethanolamine from  
Neurospora, Strain 47904 × 38706*

Experiment No	N-Methyl carbon		Carbons 1 and 2 of ethanolamine 2 carbon residue	
	Specific activity, c p m per mg carbon × 10 <sup>-6</sup>	Per cent of original specific activity in methyl of methionine*	Specific activity, c p m per mg carbon	Per cent of original specific activity in methyl of methionine
I	2 07	12 7	380	0 023
II	2 90	17 8	990	0 061

\* The specific activity in the methyl group of the administered L methionine CH<sub>3</sub>-<sup>14</sup>C was  $1.63 \times 10^6$  c p m per mg of carbon

series of reactions in which formate carbon becomes a labile methyl carbon It has not been possible in many experiments to state with certainty whether the first appearance of a newly synthesized methyl is in choline, in methionine, or in a thetin The efficient transfer of specific activity from formate to the methyl of monomethylethanolamine suggests that the formation of monomethylethanolamine from ethanolamine involves a reaction in which a formate carbon becomes a labile methyl group These findings are not in disagreement with the observed appearance of doubly labeled methyl (C<sup>14</sup> and deuterium) in tissue choline following the administration

in rats of similarly labeled methionine (16), since it is now obvious that the three successive methylations involved in the conversion of ethanolamine into choline need not involve the same precursors. Also of interest is the work of Berg (17) in which it was shown that under anaerobic conditions guinea pig liver slices convert formate to the methyl group of methionine without choline and betaine as obligatory intermediates in this conversion. Berg's studies must involve an entirely different metabolic pathway for the conversion of formate to a labile methyl from that involved in the studies presented here.

#### SUMMARY

Two double mutants of *Neurospora crassa*, 47904  $\times$  C-24 and 47904  $\times$  38706, were cultured on media containing formate- $C^{14}$  and L-methionine- $CH_3^{14}$ , respectively. The monomethylethanolamine isolated from such cultures was assayed for isotope activity. The activity incorporated in the methyl moiety of this amine was much greater from formate than from the methyl group of methionine. These findings suggest that formate is a more direct precursor of the methyl of monomethylethanolamine than is the methyl group of methionine.

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# THE DEOXYRIBONUCLEASE OF RAT LIVER IN RELATION TO THE ISOLATION OF DEOXYRIBONUCLEOPROTEIN\*

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The deoxyribonuclease of various tissues is not only intrinsically interesting, but this enzyme must be considered in the development and application of procedures for the isolation of undegraded deoxyribonucleic acids and nucleoproteins. For this purpose it is particularly desirable to know the intracellular distribution of DNase<sup>1</sup> and to have information concerning factors which modify the enzymatic activity. The intracellular distribution of DNase has been studied (1-9) in various tissues of several animals by a number of methods, with some variation in conclusions. A part of this variation in results doubtless represents real differences in the distribution of DNase with respect to the type of tissue and the species of animal, but it seems likely that some of the variability can be attributed to artifacts which arise from transposition of the enzyme from one particulate fraction to another, a source of error which may be more serious with one method of fractionation than another. Schneider and Hogeboom (5) have reported that, in homogenates of mouse liver in 0.25 M sucrose, DNase is concentrated predominantly in mitochondria. Kuff and Schneider (8) and de Duve *et al.* (9) have found high specific activities of DNase in mitochondrial fractions of rat liver isolated in 0.25 M sucrose, but the latter authors have concluded that the DNase actually is contained in a special type of granule to which they have given the name lysosome. It is not known whether the distribution of DNase of rat liver is altered in the presence of calcium chloride which is added to 0.25 M sucrose to yield a homogenizing medium which is reported (10, 11) to have particular advantages for the isolation of nuclei. It is important to have this information in order to permit isolation of nuclei under conditions which will provide minimal opportunity for DNase action on the DNP.

Maver and Greco (12, 13) reported that the DNase of calf spleen and

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<sup>1</sup> The following abbreviations are used in this paper: DNA = sodium deoxyribonucleate, DNA-P = deoxyribonucleic acid phosphorus, DNP = deoxyribonucleoprotein, DNase = deoxyribonuclease.

thymus has optimal activity at pH 4.5 and thus differs markedly from the DNase of pancreas. Siebert *et al* (3) found that there are considerable variations in the pH optima of the DNases of various organs and tissues in different species of animals, but they did not give the value for rat liver. Greenstein *et al* (14) reported that the DNase activities of several tissues are decreased by dialysis of the extracts, and the activities are restored in varying degrees by the addition of inorganic salts. Webb (7) also commented upon the effects of inorganic salts upon the activity of the DNase of calf thymus, but a systematic study of the salt effects over a wide range of concentrations and with a variety of salts seems desirable in order to determine whether the effects are attributable to general ionic strength changes or to specific stimulations and inhibitions by certain ions.

The present work is concerned with a study of the distribution of DNase in cell fractions of rat liver in sucrose- $\text{CaCl}_2$  medium and with an investigation of the effects of various inhibitors and of changes in pH and ionic strength. These studies provide information which can serve as a guide in the isolation of nuclei under conditions which minimize opportunity for changes in DNA and DNP by DNase action.

### *Methods and Materials*

Albino rats, obtained from Carworth Farms, Inc., and maintained on a diet of Purina dog chow, were fasted for 16 hours prior to removal of the livers. To determine the distribution of DNase, the livers were perfused *in situ* with ice-cold 0.15 M NaCl followed by 0.25 M sucrose containing 0.0018 M  $\text{CaCl}_2$ , and homogenization and centrifugal fractionation in 0.25 M sucrose-0.0018 M  $\text{CaCl}_2$  were performed at 2° essentially as described by Schneider and Hogeboom (15) in the procedure which involved the use of 0.25 M sucrose without  $\text{CaCl}_2$ . Nuclear (Nw), mitochondrial (Mw<sub>2</sub>), and supernatant (S<sub>1</sub>) fractions were obtained (15). DNase activity was determined by spectrophotometric measurement of the production of acid soluble compounds from highly polymerized DNA<sup>2</sup> by a modification of the method of Schneider and Hogeboom (5). Assays of cell fractions were performed at 37° in acetate buffer at pH 5.1 and ionic strength ( $\Gamma/2$ ) 0.2 with a DNA concentration of 0.33 mg per ml of the digestion mixture. Magnesium was omitted since this metallic ion was not found to be required by the acid DNase of rat liver. The digestion mixtures were oscillated gently and continuously in order to prevent sedimentation of particulate fractions during the digestion periods, and samples were removed at 0, 0.5, 1, and 4 hours<sup>3</sup>. Protein and DNA were precipitated immediately.

<sup>2</sup> DNA was isolated from calf thymus by the method of Kay, Simmons, and Dounce (16).

<sup>3</sup> When the DNase activities were low, the digestions were followed at intervals.

with cold 0.5 N perchloric acid. After centrifugation, the optical densities of the supernatant fluids were determined at  $260\text{ m}\mu$  in a Beckman spectrophotometer, model DU. The optical densities, corrected for the blank value at zero time, were directly proportional to the amount of homogenate added and to the time of incubation. Specific DNase activities are expressed in terms of the change in optical density, referred to the original digest, at  $260\text{ m}\mu$  ( $\Delta D_{260}$ ) per 30 minutes per mg of nitrogen per ml. For the preparation of buffers, substrate solutions, and homogenates for DNase assay, the distilled water was passed through a mixture of cation and anion exchangers<sup>4</sup> to remove traces of inhibitory ions (probably cupric ions) which were present in the distilled water.

In the studies on the DNP of nuclei, comparisons were made of the methods of Hogeboom *et al* (11), Dounce (17), and Dounce *et al* (18) for the isolation of nuclei. Separation of the DNP into a fraction which was soluble in 0.05 M sodium citrate and one which was not soluble gave an indication of the extent of alteration of DNP which occurred during the isolation of nuclei and during subsequent periods of incubation in sucrose solution. In the incubation experiments, nuclei isolated by the method of Hogeboom *et al* (11) were washed four times, by means of the procedure described by these authors, for the removal of mitochondria and other cytoplasmic fractions, and the washed nuclei then were suspended in 45 ml of cold 0.25 M sucrose containing 0.00018 M  $\text{CaCl}_2$ . The suspension was divided into four 10 ml portions in 15 ml centrifuge tubes. Each portion contained nuclei equivalent to those in 200 mg of fresh liver. Two tubes were centrifuged immediately at  $800 \times g$  for 10 minutes to sediment the nuclei. These nuclei were analyzed immediately for total DNA-P and "citrate-insoluble DNA-P" as described below. The other tubes were stoppered tightly and were placed in a horizontal position in a mechanical locker in a cold room at  $5^\circ$ . After 19 hours of gentle oscillation, the nuclei were sedimented by centrifugation, and the supernatant fluid was discarded. The nuclei from one tube in each pair were analyzed for total DNA-P by the procedure of Schneider (19) with some modifications adapted from the method of Ogur and Rosen (20). The nuclei in the other tube in each pair were suspended in 10 ml of cold 0.05 M sodium citrate at pH 7 and were disintegrated by high speed stirring for 5 minutes in a cold microcup of a Waring blender. The contents of the cup were oscillated in the cold room for 1 hour and then were transferred quantitatively

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during a 20 hour period. In these cases a drop of toluene was added to the digestion mixture to prevent bacterial growth. The slight ultraviolet absorption by dissolved toluene was adequately controlled by the zero time blanks.

<sup>4</sup>The ion exchanger was Deeminite which was used in the "deeminizer," Crystal Research Laboratories, Inc.

tively with citrate solution to a 25 ml plastic tube and centrifuged at  $15,000 \times g$  in the multispeed rotor of an International refrigerated centrifuge, model PR-1. The sediment was extracted a second time with another 10 ml portion of 0.05 M sodium citrate, and the centrifugation was repeated. The sediment then was analyzed for DNA-P to yield the "citrate-insoluble DNA-P" fraction. The values for "citrate-soluble DNA-P" were obtained by difference between the total DNA-P and the citrate-insoluble DNA-P. Direct analyses of the citrate extracts for DNA-P were unsatisfactory as a result of the presence of citrate and sucrose. However, in some cases the citrate extracts were treated with an equal volume of cold 1 N  $\text{HClO}_4$  to precipitate proteins and nucleic acids, and the precipitates were collected by centrifugation and analyzed for DNA-P by the methods (19, 20) mentioned above. The experiments on the effect of added cytoplasm upon the DNA-P of incubated nuclei were performed in a similar manner after the addition of 1 ml of the total cytoplasmic fraction of a 10 per cent homogenate of rat liver in 0.25 M sucrose.

The pancreatic DNase, which was used for comparison with rat liver DNase in a few experiments, was a crystalline preparation obtained from the Worthington Biochemical Corporation. *o*-Iodosobenzoic acid and sodium *p*-chloromercuribenzoate were obtained from the Sigma Chemical Company and were used without further purification.

#### RESULTS AND DISCUSSION

The data of Table I indicate that the distribution of DNase activity in homogenates of rat liver in 0.25 M sucrose-0.0018 M  $\text{CaCl}_2$  medium was similar to that reported (5, 9) for homogenates in 0.25 M sucrose without  $\text{CaCl}_2$ , *viz.*, the DNase activity was concentrated principally in the mitochondrial fraction. However, the specific DNase activity of the mitochondrial fraction isolated in sucrose alone was somewhat greater than that obtained with mitochondria isolated in sucrose- $\text{CaCl}_2$ , probably as a result in the latter case of contamination of mitochondria with microsomes which tend to aggregate and precipitate in media which contain calcium (11). From the viewpoint of the principal theme of the present paper, it is important to note that the DNase activity of the nuclear fraction was low. Counts of nuclei, whole cells, and mitochondria by the technique of Shelton *et al.* (21) indicated that the nuclear fraction contained approximately 5 per cent of the total mitochondria of the homogenate, and the ratio of free nuclei to whole cells was 45:1. Consequently, the major part of the DNase activity of the nuclear fraction could be attributed to the presence of whole cells and mitochondria, and it can be concluded that the nuclei themselves probably are devoid of DNase activity. Samples of nuclei which were washed repeatedly, by suspension in 0.25 M

ucose-0.00018 M  $\text{CaCl}_2$  and centrifugation through a layer of 0.34 M sucrose-0.00018 M  $\text{CaCl}_2$  as described by Hogeboom *et al.* (11), were found to have very small specific DNase activities (values of 0.5 to 1.8 in different experiments). The DNase activity of the supernatant fraction was only 1 per cent of the total activity of the homogenate, and at least a portion of this activity may have resulted from the release of soluble DNase from some of the mitochondria which were broken during homogenization. The DNase activity of the supernatant fraction was only slightly greater in homogenates with sucrose- $\text{CaCl}_2$  than in those prepared with sucrose

TABLE I

*Distribution of DNase Activities in Fractions Obtained from Rat Liver Homogenates in 0.25 M Sucrose 0.0018 M  $\text{CaCl}_2$*

The values reported are for 100 mg of perfused liver or an equivalent amount of fraction, and they are the averages of the assays and analyses of three homogenates. DNase activities were determined at 37° in acetate buffer at pH 5.1 and strength 0.2.

Liver fraction	Total nitrogen	DNase activity		
		Total	Fraction of homogenate	Specific
	mg	$\Delta D_{260}$ per 30 min	per cent	$\Delta D_{260}$ per 30 min per mg N per ml
Homogenate	2.55	14.3	(100)	5.6
	0.252	0.98	6.9	3.9
	0.726	10.8	75.5	14.9
	1.53	1.96	13.7	1.3
Supernatant			96.1	

upture of mitochondria by freezing and lyophilization resulted in an increase in specific DNase activity of this fraction (from 15 to 26 units, Table I). DNase is readily extracted by 0.15 M NaCl or 0.05 M Na citrate from fragmented mitochondria. Schneider and Hogeboom have reported similar increases in the DNase activity of mouse liver mitochondria as a result of sonic disintegration, and de Duve *et al.* (9) have suggested various methods for the release of DNase from mitochondria (lysosomes). Lyophilized mitochondria and nuclei were used in the experiments on the effects of pH and ionic strength upon DNase activity (Tables 1 and 2) and in the studies on inhibitors (Table II). Optimal activity was noted at pH 5.1 with both nuclear and mitochondrial preparations. However, with both preparations a slight secondary rise in activity

was observed at pH 6.8 to 7.3, and in this range of pH the activity was increased by magnesium ions ( $1 \times 10^{-3}$  M) in contrast to the absence of any stimulatory effect by this ion at pH 5. The DNase activity at pH 7 was abolished by citrate ( $1 \times 10^{-2}$  M) or ethylenediaminetetraacetate ( $1 \times 10^{-3}$  M), but the activity at pH 5 was not inhibited by these chelating agents.

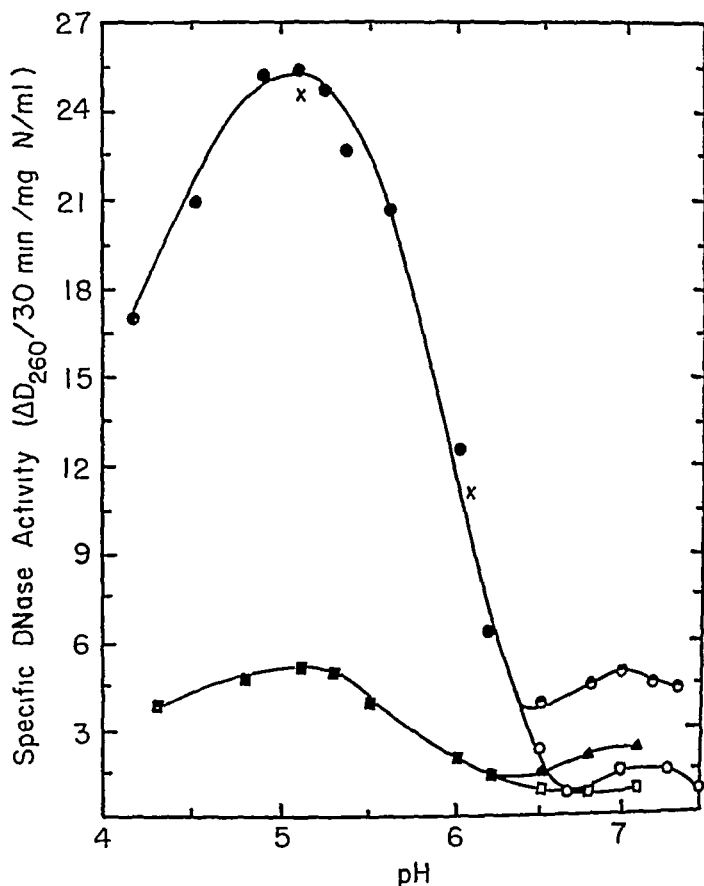


FIG. 1 Effect of pH upon the DNase activity of nuclei and mitochondria of rat liver. ●, mitochondria in acetate buffer ( $\Gamma/2 = 0.2$ ), X, mitochondria in acetate buffer ( $\Gamma/2 = 0.2$ ), containing  $1 \times 10^{-3}$  M  $Mg^{++}$ , ○, mitochondria in phosphate buffer ( $\Gamma/2 = 0.1$ ), ⊙, mitochondria in phosphate buffer ( $\Gamma/2 = 0.1$ ), containing  $1 \times 10^{-3}$  M  $Mg^{++}$ , ■, nuclei in acetate buffer ( $\Gamma/2 = 0.2$ ), □, nuclei in phosphate buffer ( $\Gamma/2 = 0.1$ ), ▲, nuclei in phosphate buffer ( $\Gamma/2 = 0.1$ ), containing  $1 \times 10^{-3}$  M  $Mg^{++}$ .

unless they were added in amounts which were large enough to yield an unfavorable ionic strength. It seems probable that there are two different DNase enzymes in rat liver, an "acid" DNase which does not require magnesium ions and a "neutral" DNase which does. Cunningham and Laszkowski (22) have reported the presence of an acid and a neutral DNase in veal kidney, and Allfrey and Mirsky (6) have found both enzymes in pancreas.

The acid DNase activity of rat liver is markedly dependent upon ionic strength (Fig 2) At pH 5 in either acetate or citrate buffers the specific activity is very slight at low ionic strength, it increases to a maximum at

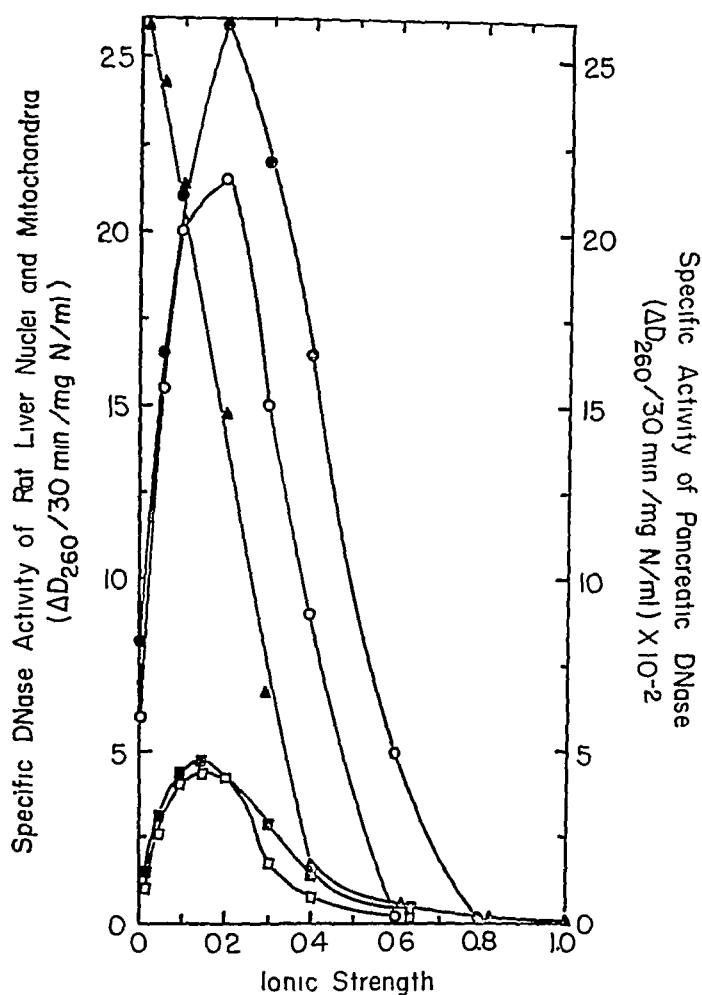


Fig 2 Effect of ionic strength upon the DNase activity of nuclei and mitochondria of rat liver and upon the activity of crystalline DNase of calf pancreas mitochondria in acetate buffer, pH 5,  $\circ$ , mitochondria in citrate buffer, pH 5, nuclei in acetate buffer, pH 5,  $\square$ , nuclei in citrate buffer, pH 5,  $\blacktriangle$ , crystalline pancreatic DNase (5  $\gamma$  per ml) in phosphate buffer (0.016 M), pH 7, 0.0033 M  $Mg^{++}$ , ionic strength varied with NaCl

ionic strength 0.15 to 0.2 and then declines at higher values and is practically zero above ionic strength 0.8. Values are slightly lower in citrate than in acetate buffers, but the specific effect of citrate ion is small. Activity-ionic strength curves similar to those shown in Fig 2 were obtained when the ionic strength was varied with sodium chloride, potassium chloride, or potassium sulfate. The variations in activity appear to be



attributable to changes in ionic strength rather than to specific effects of certain ions. In contrast to the acid DNase of liver, the neutral DNase of calf pancreas has maximal activity at low ionic strength, and the activity declines almost linearly with increasing ionic strength (Fig 2). Similar results with pancreatic DNase were reported by Kunitz (23) from measurements which were restricted to a more limited range of ionic strength.

Maver and Greco (13) reported that the DNase of calf spleen and thymus is inhibited by *p*-chloromercuribenzoate. Webb (7) found little inhibition of calf thymus DNase by sodium *o*-iodosobenzoate. The data of Table II indicate that the acid DNase activity of rat liver mitochondria is decreased to less than 50 per cent of control values by *p*-chloromer

TABLE II  
*Effect of Various Inhibitors upon DNase Activity of Rat Liver Mitochondria at 37° and pH 5 in Acetate Buffer*

$\Gamma/2 = 0.15$

Inhibitor	Specific activity
<i>M</i>	$\Delta D_{260}$ per 30 min per mg N per ml
None	25.7
<i>o</i> -Iodosobenzoate, $1 \times 10^{-4}$	23.9
“ $1 \times 10^{-3}$	22.0
<i>p</i> -Chloromercuribenzoate, $5 \times 10^{-4}$	17.8
“ $1 \times 10^{-3}$	10.2
Cystine, $1 \times 10^{-3}$	23.8
Cu <sup>++</sup> , $1 \times 10^{-4}$	11.6
“ $1 \times 10^{-3}$	3.4
“ $1 \times 10^{-2}$	0.4

curibenzoate at a concentration of  $1 \times 10^{-3}$  M, but the activity is only slightly affected by similar concentrations of cystine and *o*-iodosobenzoate. Thus, it is questionable whether the activity of the acid DNase of rat liver is dependent upon thiol groups, but judgment should be reserved until similar studies are made with the isolated enzyme. Webb (7) reported that calf thymus DNase is strongly inhibited by cupric ions at 0.01 M. The acid DNase activity of rat liver mitochondria also is very sensitive to cupric ions (Table II).

The data of Tables III and IV indicate that in freshly prepared nuclei an appreciable percentage of the total DNA-P is soluble in 0.05 M sodium citrate. Inasmuch as highly polymerized, fibrous preparations of DNP of rat liver are practically insoluble in 0.05 M sodium citrate, it seems probable that the DNA-P of rat liver nuclei which can be extracted with this solvent may correspond to a partially degraded DNP which is produced by

DNase action during the isolation of the nuclei. This conclusion is supported by the observation (Table III) that the citrate-soluble DNA-P increases in amount during the incubation of the nuclei at 5°, particularly when cytoplasmic fractions are added to the medium. A considerable fraction (30 to 80 per cent in different experiments) of the citrate-soluble DNA-P can be precipitated from the citrate extracts with cold 0.5 N HClO<sub>4</sub>, consequently, the extent of the degradation may not be great. However, the degradation which occurred probably involved the DNA portion of the DNP, since otherwise the DNA would have been almost completely precipitable by acid from the citrate extracts. This conclusion has been con-

TABLE III

*Increase in Citrate-Soluble DNA-P during Incubation of Rat Liver Nuclei at 5°*

Averages of four experiments. Each incubation mixture contained nuclei equivalent to those in 200 mg of liver. The nuclei were isolated by the method of Hogboom *et al.* (11).

Incubation mixture	Duration of incubation	Total DNA-P	Citrate-insoluble DNA-P*	Citrate-soluble DNA-P (by difference)	
	hrs	γ	γ	γ	per cent of total
Nuclei in 10 ml of 0.25 M sucrose-0.00018 M CaCl <sub>2</sub> , pH 6.4	0	39.2	33.3	5.9	15
	19	38.3	29.5	8.8	23
Nuclei in 10 ml of 0.25 M sucrose-0.00018 M CaCl <sub>2</sub> , with 1 ml of cytoplasmic fraction,† pH 6.4	0	39.6	32.9	6.7	17
	19	33.4	18.4	15.0	45

\* DNA-P which was not extracted from disintegrated nuclei by 0.05 M sodium citrate at pH 7.

† Cytoplasmic fraction of a 10 per cent homogenate of rat liver in 0.25 M sucrose

used by isolation from the citrate extracts of a deoxypentose polynucleotide of low intrinsic viscosity. However, the possibility has not been excluded that some degradation of the protein portion of the DNP may have occurred also. In this connection it should be mentioned that de Duve *et al.* (9) have reported that lysosomes contain a major part of the cathepsin activity, as well as the DNase activity, of sucrose homogenates of rat liver. The formation of the citrate-soluble DNA-P appears to be more rapid during the early stages of the isolation of the nuclei than during subsequent periods of incubation of the nuclei in sucrose-CaCl<sub>2</sub> medium in the absence of added cytoplasmic fractions. Thus, the citrate-soluble DNA-P was 15 per cent of the total DNA-P immediately after the nuclei were isolated and washed, and it was only 23 per cent of the total after 19 hours of incubation at 5° in the absence of cytoplasmic fractions (Table I). It seems possible that a portion of the citrate-soluble DNA-P pre-

exists within the nucleus prior to homogenization. However, inasmuch as the DNase of rat liver is located principally within the mitochondria (or lysosomes (9)), the DNP is most subject to attack by this enzyme during the homogenization and during the interval prior to completion of the first centrifugation for the separation of nuclei from cytoplasmic fractions. For the preservation of the DNP it is essential to homogenize the liver by procedures which will cause minimal trauma to mitochondria and to nuclei. We have found that prolongation of the period of homogenization beyond the 2 minute interval recommended by Hogeboom *et al* (11) increases the citrate-soluble DNA-P. The medium for homogenization

TABLE IV

*Average Content of Total, Citrate-Insoluble, and Citrate-Soluble DNA P in Nuclei Isolated from Rat Liver by Various Methods*

The values are the averages of four determinations by each method except the first, in which six determinations are represented

Medium for homogenization	pH of homogenate	DNA-P per nucleus (mg $\times 10^{-3}$ )		
		Total	Citrate insoluble*	Citrate-soluble (i.e., difference)
0.25 M sucrose-0.0018 M $\text{CaCl}_2$ †	6.4	8.4	6.3	2.1
0.25 " sucrose-0.0018 " " pH 7.0	6.8	7.6	6.5	1.1
Citric acid, pH 6‡	6.0	7.8	3.6	4.2
0.44 M sucrose-citric acid, pH 6.2§	6.2	7.2	5.3	1.9

\* DNA-P which was not extracted from disintegrated nuclei by 0.05 M sodium citrate at pH 7

† Method of Hogeboom, Schneider, and Striebig (11)

‡ Procedure of Dounce (17)

§ Method of Dounce *et al* (18)

should be one which causes minimal damage to mitochondrial and nuclear membranes and which provides an unfavorable pH for the action of acid DNase. In our hands, the medium described by Hogeboom *et al* (11) was quite satisfactory for this purpose, particularly when the medium was adjusted to an initial pH of 7 (Table IV). Homogenization in dilute citric acid (17) at pH 6 yielded nuclei with a high percentage of citrate-soluble DNA-P (Table IV). This medium damages mitochondria (18) and provides a pH which is favorable for some DNase activity. Dounce and Monty (24) have reported that nuclei isolated from homogenates in citric acid at pH 6, or in 0.25 M sucrose with use of the Waring blender, fail to form gels in dilute alkali, and they have attributed this to a change in the DNP which occurs during the homogenization and isolation of the nuclei. Dounce *et al* (18) concluded that this change in the DNP re-

sulted from the action of a mitochondrial enzyme which may not be DNase. However, it appears that the possibility of some DNase action was not excluded. Nuclei isolated from rat liver homogenates by the recently described method of Dounce *et al* (18) are capable of forming gels in dilute alkali (18, 24), and they contain only small percentages of citrate-soluble DNA-P (Table IV). It has been our experience that nuclei isolated from rat liver in 0.25 M sucrose-0.0018 M  $\text{CaCl}_2$  (11) also form gels in dilute alkali.

If nuclei are to be used for the isolation of DNP, it is not only important to avoid damaging nuclear and mitochondrial membranes and to maintain conditions which are unfavorable for the activity of DNase, but also essential to wash the nuclei repeatedly in fresh medium to remove residual mitochondria and to observe additional precautions during subsequent extraction of DNP. For example, we have found significant DNase activity in preparations of nuclei (11) after four washings in sucrose- $\text{CaCl}_2$ . This residual DNase is almost completely extracted by cold 0.05 M sodium citrate at pH 7 when the nuclei are disintegrated by a brief period of high speed homogenization in this medium for the removal of other proteins prior to extraction of DNP with 1 M NaCl. Consequently, the duration of the first extraction with 0.05 M sodium citrate should be brief to minimize exposure of the DNP to DNase action. Fortunately, the pH and ionic strength of the 0.05 M sodium citrate solution are unfavorable for acid DNase action, and citrate inhibits neutral DNase activity. Therefore, citrate solution is preferable to 0.15 M NaCl for the extraction of other proteins prior to extraction of DNP. After removal of residual DNase and other extraneous proteins by several extractions with 0.05 M citrate, a portion, at least, of the DNP can be extracted from the citrate-insoluble residue with 1 M NaCl and isolated by the general procedure of Mirsky and Pollister (25) as modified by Petermann and Lamb (26). Any residual DNase of either type would be inactive during extraction of DNP with 1 M NaCl by reason of the high ionic strength (Fig. 2). With such precautions we have been able to obtain from rat liver nuclei, isolated in sucrose- $\text{CaCl}_2$ , fibrous preparations of DNP which yielded high values of specific viscosity in 1 M NaCl. For example, one preparation of DNP in 1 M NaCl gave a value of 610 for  $\eta_{sp}$  (P) (27) at 25° for a concentration of 15  $\gamma$  of DNP phosphorus per ml. The N/P ratio (by weight) was 3.65 and  $\epsilon$  (P) (28)  $\times 260 \text{ m}\mu = 6700$ . This preparation of DNP involved a 12 hour period of extraction of the disintegrated nuclei with 1 M NaCl at 5°. Approximately 35 per cent of the total DNA-P remained unextracted from the nuclear fragments at the end of this period. Dounce *et al* (18) have called attention to the difficulty involved in the extraction of DNP from nuclei which have been protected from the action of enzymes. On the other

hand, we have confirmed the observations of Luck *et al* (29) that rat liver nuclei isolated in dilute citric acid at pH 6 yield non-fibrous preparations of DNP with relatively low values of specific viscosity. The DNP is more readily extracted by 1 M NaCl from nuclei isolated in dilute citric acid than from those obtained from sucrose-CaCl<sub>2</sub> homogenates. Further work obviously is required to determine which type of DNP preparation corresponds to "native" DNP. The problem of degradation of DNP and DNA during isolation has great bearing upon current efforts to study the heterogeneity of these macromolecules, since even a slight degree of degradation could produce considerable heterogeneity.

#### SUMMARY

The major portion of the deoxyribonuclease (DNase) activity of rat liver was found to be associated with the mitochondrial fraction of homogenate prepared in sucrose-CaCl<sub>2</sub>, as well as those obtained with 0.25 M sucrose by the method of Schneider and Hogeboom. For the principal DNase of rat liver, a pH optimum of 5.1 was observed, and the activity at this pH was not increased by low concentrations of magnesium ions. At this pH the DNase activity was slight at low ionic strength, it reached a maximum at ionic strength 0.15 to 0.2 and then declined at higher values and was practically zero above ionic strength 0.8. A slight secondary rise in DNase activity was noted at pH 6.8 to 7.3, and in this range of pH low concentrations of magnesium ions increased the activity. The DNase activity at pH 5 was inhibited by cupric ions and by *p*-chloromercuribenzoate, but was not significantly inhibited by *o*-iodosobenzoate or cystine.

Rat liver nuclei, isolated by several methods, contain a significant amount of the total deoxyribonucleoprotein (DNP) in a form which permits extraction of a portion of the sodium deoxyribonucleate (DNA) from the disintegrated nuclei with 0.05 M sodium citrate. This citrate-soluble DNA increases when the nuclei are incubated in fresh homogenizing medium at 5°, and the rate of increase is accelerated when cytoplasmic fractions are added. This fraction of the DNA was found to be partially degraded, but the possibility was not excluded that some alteration also had occurred in the accompanying protein. This criterion of degradation of DNP was utilized in a comparison of several methods for the isolation of nuclei in relation to the use of such preparations as starting material for the isolation of DNP.

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# STUDIES ON THE NATURE OF THE AMINO ACID INCORPORATION PROCESS OF HEN OVIDUCT TISSUE

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A number of recent investigations have been concerned with the relative localization of ribonucleic acid and of protein-synthetic processes within specific structural components of cells (1-9). The results thus far have shown that, after incorporation of radioactive amino acids *in vivo* or *in vitro*, the radioactivity is concentrated mainly in the small cytoplasmic particles called microsomes. Preparations of isolated microsomes have also been shown to be most active in incorporating labeled amino acids. The microsomes also account for most of the ribonucleic acid (10-13) of the cells, and this correspondence of ribonucleic acid content and amino acid-incorporating ability is in accord with a proposed relationship between these two properties (1, 2).

The present paper describes experiments performed with a tissue highly specialized with respect to protein synthesis, the oviduct of the laying hen. These results also demonstrate a relationship between amino acid-incorporating ability and ribonucleic acid content, but, in contrast to other tissues studied thus far, microsome-like material in this tissue has been found to sediment in low centrifugal fields ( $<600 \times g$  in less than 10 minutes as opposed to  $20,000 \times g$  or greater for 1 hour or longer (13)). Chemical and cytochemical examinations have shown that the bulk of the cytoplasmic basophilia of the cells is contained in this sedimenting fraction.

It has been determined further that this fraction has the greatest tendency to lose previously incorporated amino acids when the specific activity of the free amino acid pool is lowered during the incubation. In contrast, the more soluble proteins continue to increase in specific activity during the period of incubation in the medium in which the specific activity is lowered.

## EXPERIMENTAL

*Tissue System, Medium, and Incubation*—Hen oviducts obtained from laying hens were minced with scissors in the cold and incubated in a gas-

\* Part of this work was performed as a Fellow of The National Foundation for Infantile Paralysis.



equilibrated medium (95 per cent  $O_2$ -5 per cent  $CO_2$ ) (14) which had the following final concentrations  $NaCl$  0.033 M,  $KCl$  0.072 M,  $NaHCO_3$   $NaHC^{14}O_3$  0.040 M, glucose 2 gm per liter, and phenol red indicator solution 2 ml per liter.  $C^{14}O_2$  was added to the gas-equilibrated medium as  $NaHC^{14}O_3$ . 5 gm of tissue were gently shaken with 12.5 ml of medium at 37° for 2 hours unless otherwise stated.

*Fractionation without Sucrose*—After incubation, the suspension was centrifuged at about 1000 to 2000 r.p.m. and washed once with water. The soluble extracellular proteins were precipitated from the supernatant fluid by the addition of alcohol to 70 per cent. The washed tissue mince was homogenized for two 2 minute periods with half its volume of distilled water in a Potter-Elvehjem glass homogenizer which was immersed in an ice bath. Microscopic examination has shown that at least 90 per cent of the cells was broken by this procedure.<sup>1</sup> The homogenate was centrifuged at about 1000 to 2000 r.p.m. for 2 minutes. The pellet was washed three times with distilled water and is referred to here as the cell debris (CD). The supernatant fluid obtained by centrifuging the homogenate was adjusted to pH 5 with 1 M acetic acid and brought to 40 per cent saturation with solid ammonium sulfate (AMS) (40 per cent AMS precipitate). The proteins soluble in 40 per cent ammonium sulfate were precipitated by the addition of trichloroacetic acid (TCA) to about 8 per cent final concentration. Pure egg albumin was isolated by isoelectric crystallization from the 40 per cent AMS-soluble fraction (14). This protein was then crystallized three times after the addition of carrier. Plakalbumin was obtained from the egg albumin by enzymatic degradation with *Bacillus subtilis* enzyme (14).

*Fractionation with Sucrose*—The tissue was minced either in the buffer used for incubation or in ice-cold 0.25 M sucrose. In the former case, excess buffer was removed by centrifugation, and in the latter case a separate sucrose wash was used to exclude all traces of external salt. The tissue obtained in both instances was homogenized in about 5 volumes of ice-cold 0.25 M sucrose and fractionated by differential centrifugation as described by Schneider (13). For the tissue fractionated after incubation, the same procedure was followed with and without first washing in 0.25 M sucrose.

*Determinations of Ribonucleic Acid*—1 ml aliquots of suitably diluted homogenate and of the various protein fractions described above were precipitated with 1 ml of cold 1 N  $HClO_4$  and washed once with 2 ml of cold 1 N  $HClO_4$ . After the above precipitation and extraction, 2 ml of 1 N  $HClO_4$  and 2 ml of orcinol reagent were added to each sample, and the suspensions were boiled for 30 minutes in a water bath. The reagent was

<sup>1</sup> Hendler, R. W., and Glenner, G., unpublished data.

prepared before use by dissolving 10 mg per ml of orcinol in a stock solution of 0.4 per cent ferric ammonium sulfate in concentrated HCl (15). After removal of precipitated protein by centrifugation, the optical density was determined at 660 m $\mu$ , and the values obtained were corrected for the reagent blank.

*Preparation of Proteins for Determinations of Radioactivity*—The proteins were precipitated by adding a 10 per cent TCA solution to a final concentration of about 8 per cent, and the precipitate was washed four times in 5 per cent TCA and the second wash heated at 95° for 15 minutes. The proteins were then washed once with absolute alcohol and three times with a 3:1 mixture of alcohol and ether, with 5 minutes heating at 65° during each alcohol-ether wash. Finally, the proteins were washed once with absolute ether and then plated from an absolute ether suspension on previously weighed planchets of 1.54 sq cm area. The radioactivity of the planchet dried in air was determined with a thin window Geiger-Muller counter, sufficient counts being taken to give less than 5 per cent statistical error. Corrections were applied for self-absorption by using an empirical curve obtained for BaCO<sub>3</sub>. In several cases it was further determined that the proteins so treated retained their radioactivity after being dialyzed 24 hours at room temperature in a solution of 1 M NH<sub>4</sub>OH made up in 60 per cent dimethyl formamide.<sup>2</sup>

*Removal of Radioactivity before Continued Incubation to Lower Specific Activity of Amino Acid By Gas Exchange*—After 2 hours of incubation, the well agitated flasks were flushed with 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub>, and flushing was continued throughout the rest of the experiment.

*By Medium Exchange*—After 2 hours of incubation, the contents of the flasks were gently centrifuged (about 1000 r p m), washed in buffer, and resuspended in either unlabeled buffer, radioactive buffer, or unlabeled buffer in which hen oviduct mince had been incubated for 2 hours.

### Results

*Sedimentation of Ribonucleic Acid and Incorporated Radioactivity*—It has been consistently observed (see Tables I to III) that, in incubations of hen oviduct mince with radioactive CO<sub>2</sub>, which mainly labels glutamic and aspartic acids, and with radioactive glycine, phenylalanine, valine, and alanine, the cell debris accounts for the major fraction of incorporated amino acid. The specific activity of this material is usually appreciably higher than that of the other fractions. Since it is known that this fraction represents a mixture of proteins, it might be expected that subfrac-

<sup>2</sup> It is worth mentioning that the dialysis causing itself liberates a wide spectrum of anhydriin-reactive materials which have a maximal optical density between 270 and 280 m $\mu$ .

TABLE I  
Comparison of Fractions\* Obtained by Water and Sucrose Fractionation

	Fraction	C p m per mg	Total mg	Per cent homogen- ate by weight	Total c p m × 10 <sup>3</sup>	Per cent homogen- ate by c p m	Per cent distribu- tion of total RNA†
Sucrose‡	CD	30	363	45	10.9	68	72
	Mitochondria§	32	42	5	1.34	8	4
	Microsomes§	33	21	3	0.68	4	2
	Supernatant	8.2	385	47	3.16	20	4
Water	CD	33	304	46	10.0	61	70
	40% AMS ppt	17	348	52	6.0	37	17
	40% AMS-soluble	18.6	15	2	0.28	2	2

\* All incubations were carried out with intact cells, the fractionation being accomplished after incubation

† The ribonucleic acid (RNA) distributions were made with a portion of the mince which was not incubated

‡ The sucrose preparation was minced in a salt buffer and then, after the buffer was removed by centrifugation, was fractionated in sucrose as described in the text

§ These terms refer to the material which sedimented in fractions which, by analogy to other tissues, would be so classified, but do not imply identity to these structures from other tissues

TABLE II  
Fractions\* Obtained from Sucrose‡ with Complete Exclusion of External Salt

	Fraction	C p m per mg	Total mg	Per cent homogen- ate by weight	Total c p m × 10 <sup>3</sup>	Per cent homogen- ate by c p m	Per cent distribu- tion of total ribonucleic acid†
C <sup>14</sup> O <sub>2</sub> -incubated	CD	16	245	50	3.92	66	69§
	Mitochondria	26	36	7	0.94	16	5
	Microsomes	34	26	5	0.88	15	4
	Supernatant	6	186	38	0.23	4	6
Glycine-C <sup>14</sup> -incubated	CD	40	245	50	9.80	60	69§
	Mitochondria	87	36	7	3.13	20	5
	Microsomes	92	26	5	2.39	15	4
	Supernatant	19	186	38	0.72	4	6

\* All incubations were carried out with intact cells, the fractionations being accomplished after incubation

† Tissue was minced, washed, homogenized, and fractionated in 0.25 M sucrose as described in the text

‡ The ribonucleic acid distributions were made with a portion of the mince which was not incubated

§ After 2 hours incubation, 76 per cent appears in this fraction

|| These terms refer to the material which sedimented in fractions which, by analogy to other tissues, would be so classified, but do not imply identity to these structures from other tissues

TABLE III

*Generality of Amino Acid-Incorporating Ability of Cell Debris*

Fraction	Glycine			Phenylalanine		
	C p m per mg	Total c p m *	Per cent	C p m per mg	Total c p m *	Per cent
CD	224	17,000	60	60	4550	56
40% AMS ppt	131	7,070	25	42	2270	28
40% AMS-soluble	94	4,230	15	28	1260	15
Soluble extracellular	29	50	0.2	0.3	5	0.1
Fraction	Valine			Alanine		
	C p m per mg	Total c p m *	Per cent	C p m per mg	Total c p m *	Per cent
CD	594	45,000	54	54	4100	76
40% AMS ppt	450	24,300	29	16	865	16
40% AMS-soluble	315	14,200	17	7.5	340	6
Soluble extracellular	6.7	120	0.1	5.9	110	2

\* Based on the average weight of each fraction in this experiment, CD, 76 mg, 40 per cent AMS precipitate, 54 mg, 40 per cent AMS-soluble, 45 mg, soluble extracellular, 18 mg

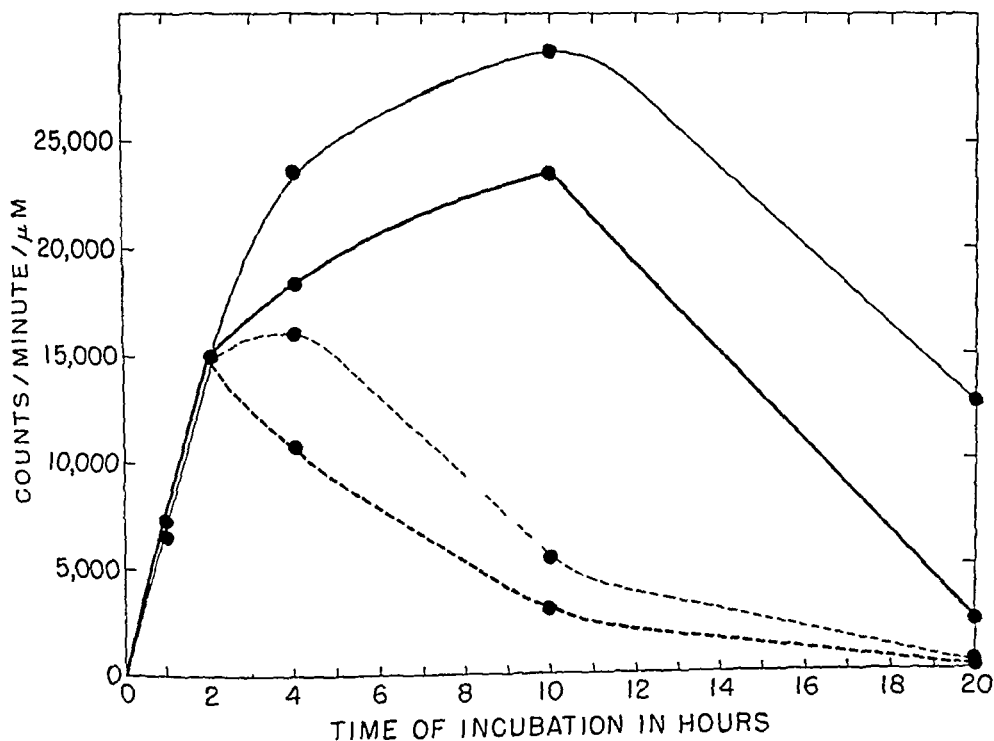


Fig 1 Specific activity of free glutamic (lighter lines) and aspartic acids (heavier lines) as a function of time of incubation of hen oviduct mince with  $C^{14}O$ . The dotted lines, which start at 2 hours, represent the specific activities of free glutamic (lighter lines) and aspartic acids (heavier lines) as a function of time during which unlabeled 95 per cent  $O_2$ -5 per cent  $CO_2$  was flushed through the medium. These data correspond to the experiments of Fig 4

tionation would yield certain components having a markedly higher specific activity than that determined for the whole fraction. It is interesting to note that, when rigid steps were taken to exclude all external salt, the material sedimenting under high speed centrifugation (microsomes, by analogy to liver) showed a higher specific activity than the average for the cell debris fraction (Table II). This microsome-like fraction, however,

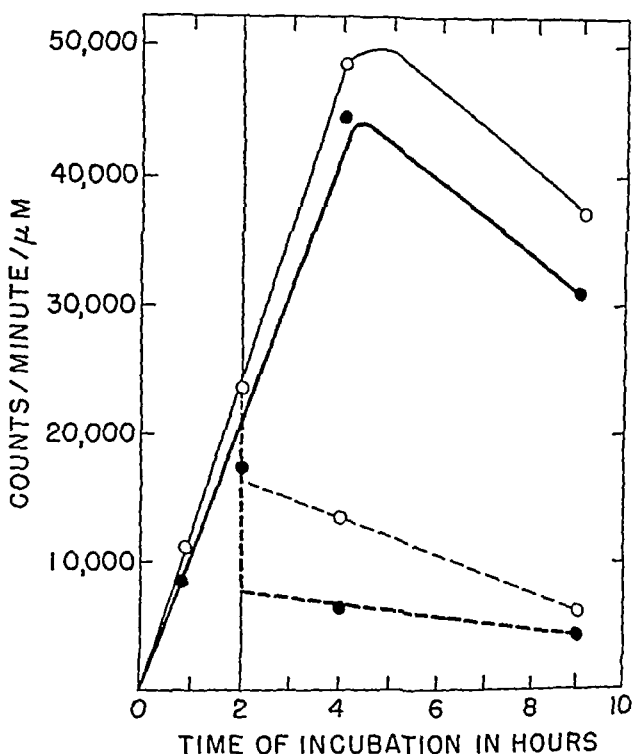


FIG 2 Specific activities of free glutamic (O) and aspartic acids (●) as a function of time of incubation of hen oviduct mince with  $C^{14}O_2$ . The dotted lines, which start at 2 hours, represent the specific activities of free glutamic (lighter line) and aspartic acids (heavier line) as a function of time after the medium was replaced at 2 hours with a fresh labeled medium for the control, and a fresh unlabeled medium for the experiment. These data correspond to the experiment of Fig 5.

represented only a small portion of the total incorporated radioactivity. These results suggest that the high specific activity of the cell debris fraction may be due in part to potentially separable "microsome-like" material.

Contrary to results obtained with other tissues, it may be seen that, in the tissues of the hen oviduct, material which corresponds in ribonucleic acid content and amino acid-incorporating ability to "microsomes" sediments easily in low centrifugal fields. The cell debris fraction contains nearly all of the ribonucleic acid, as determined by the orcinol method and

cytochemically<sup>1</sup> by affinity for basic dyes before and after ribonuclease treatment. These results were essentially independent of the method of fractionation.

The radioactivity of the cell debris proteins, obtained after incubation with  $C^{14}O_2$ , was completely localized in the amino acids, principally glutamic and aspartic acids, isolated from the hydrolyzed protein by chromatography on Dowex 50<sup>3</sup>. This radioactivity was not removed by hot TCA.

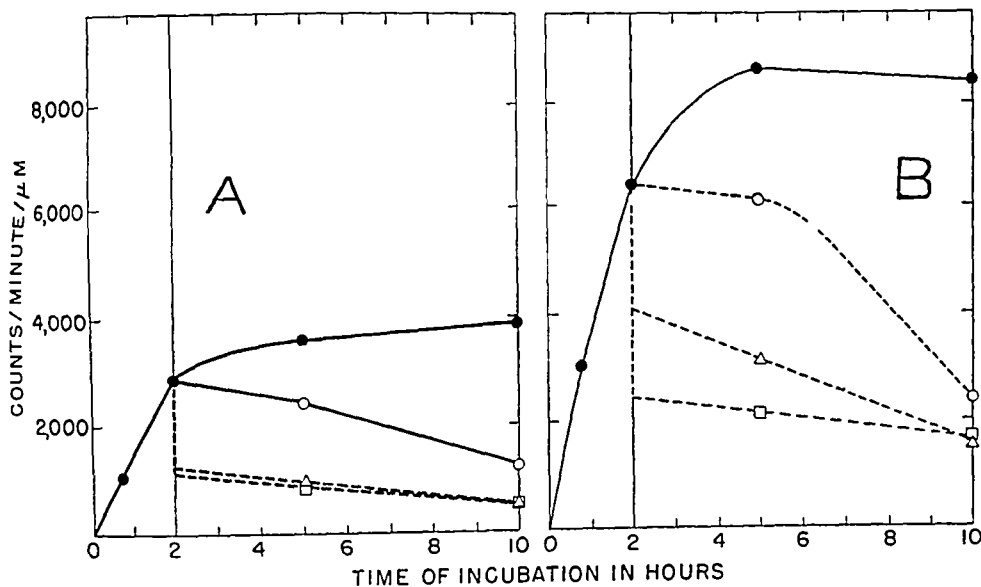


Fig 3 A, the curves represent specific activities for free (aspartic) acid as a function of time of incubation of hen oviduct mince with  $C^{14}O_2$ . The control (●) was incubated in the fully radioactive medium. The lines, which start at 2 hours, represent specific activities of aspartic acid as a function of time, during which unlabeled  $CO_2$  was passed through the medium (○), and after the medium was replaced at 2 hours with a fresh unlabeled medium (△), and an unlabeled 2 hour incubated medium (□). B, information is the same for glutamic acid as under A for aspartic acid. These data correspond to the experiment of Table IV.

or warm alcohol-ether, or by dialysis for 24 hours against 60 per cent dimethyl formamide in 1 N  $NH_4OH$ .

That the radioactive amino acids in the protein fraction were not merely physically adsorbed was further indicated by the observation that, in the free amino acid pool, the total and specific radioactivity of glutamic acid was greater than that of aspartic acid, whereas in the protein hydrolysate this situation was reversed.

#### *Amino Acid-Incorporating Activity of Cell Debris Fraction in Relation to*

<sup>3</sup> The author is indebted to Dr. David R. Kominz of the National Institute of Arthritis and Metabolic Diseases for performing this fractionation.

*That of Other Fractions*—The rates of labeling of the free glutamic and aspartic acids during the incubations are shown in Figs 1, 2, and 3. Figs 4 and 5 and Table IV show the corresponding kinetics of labeling for each protein fraction.

An initial lag period in the rate of labeling is generally noticeable for the ovalbumin (or plakalbumin) and extracellular protein fraction. This lag resembles that described by Peters (16) and attributed by him to the

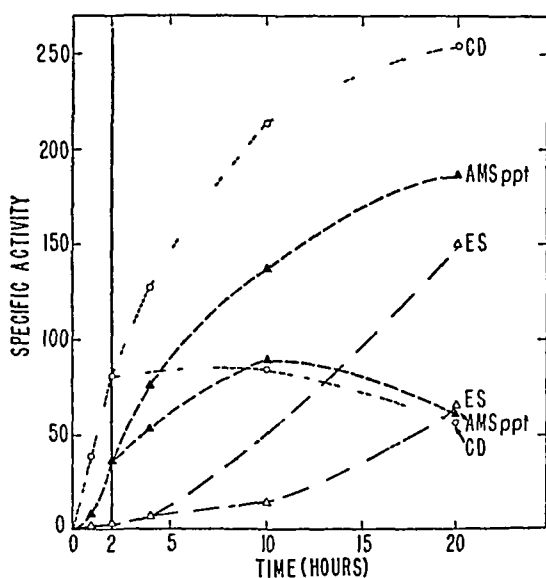


Fig. 4. Three protein fractions: O, the cell debris (CD), ▲, the proteins precipitated at pH 5 by 40 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$  (AMS precipitate), △, the extracellular soluble proteins (ES). At 2 hours, the specific activity of the free amino acid pool was lowered by bubbling unlabeled  $\text{CO}_2$  through the medium. See Fig. 1 for the specific activities of the free amino acids. The branching at 2 hours represents a control (upper branch) and experimental (lower branch) fraction obtained after incubation in fully radioactive medium and medium of reduced specific activity, respectively.

possible passage of label through precursors before entering these proteins.<sup>4</sup> A lag is not observed for the cell debris proteins and only slightly for the fraction precipitable by 40 per cent of ammonium sulfate saturation. In those cases in which incubation was continued after the specific activity of the amino acid pool had been lowered, it may be seen (Figs 4 and 5 and Table IV) that the cell debris proteins were affected the most, and that they actually decreased in specific activity in four out of the five experimental cases studied. On the other hand, amino acid incorporation continued at an appreciable rate into the ovalbumin and extracellular

<sup>4</sup> See also Ziegler and Melchior (8).

protein fraction, as reflected by the increase in specific activity of these fractions during incubation in the medium of lowered specific radioactivity

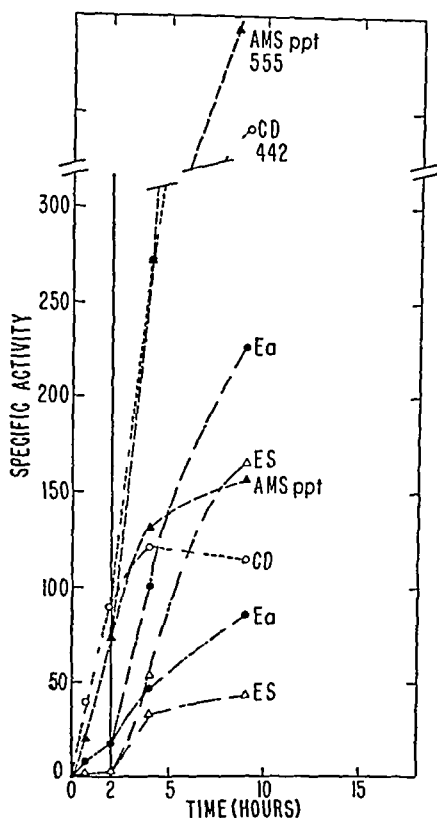


FIG 5 Four protein fractions, of which three are identical to the ones in Fig 4 and the fourth is pure crystalline egg albumin ( $E\alpha$ ). At 2 hours the specific activity of the free amino acid pool was altered by replacing the medium with a fresh  $C^{14}O_2$ -containing medium for the control and a fresh  $C^{12}O_2$ -containing medium for the experimental fraction. See Fig 2 for the specific activities of the free amino acids. The curves swing abruptly up at 2 hours and the AMS precipitate and CD curves cross at 5 hours because the diluting bulk of soluble proteins was removed at 2 hours. The branching at 2 hours represents a control (upper branch) and an experimental (lower branch) fraction obtained after incubation in fully radioactive medium and medium of reduced specific activity, respectively.

#### DISCUSSION

The correlation between the ribonucleic acid content and the ability to incorporate radioactive amino acids, which has been found for other tissues, is also apparent in the oviduct. However, it is shown that, although the material previously demonstrated to account for these characteristics was sedimented only with difficulty in relatively high centrifugal fields



(>20,000  $\times g$ ), corresponding material in this tissue sediments quite easily (<600  $\times g$  in a few minutes)

The possibility cannot be eliminated that, in this tissue, some hitherto undescribed process is responsible for the artificial clumping of microsomes out of solution. External salt was eliminated as a possible clumping agent by the fractionations in sucrose. Removal of the connective tissue before fractionation did not alter the basic distribution pattern of ribonucleic acid and radioactivity. The pH of the albumin-secreting region of the oviduct

TABLE IV  
*Rate of Incorporation of Radioactivity into Different Protein Fractions  
and Effect of Reducing Specific Activities of Precursors*

Time	Cell debris	40% AMS ppt	Plakalbumin	Extracellular supernatant proteins
<i>min</i>				
45	44*	38	9	0
<i>hrs</i>				
2†	145	128	35	4
5	355	273	110	17
10	624	588	246	53
5‡ (Gas)	267	248	73	21
10	205	369	102	46
5‡ (Medium A)	147	142	52	15
10	170	200	61	25
5‡ (Medium B)	140	134	62	
10	132	229	86	

\* All the figures are in counts per minute per mg of protein

† Starting at 2 hours, unlabeled (95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub>) gas was passed through the remaining flasks in the gas exchange experiment. In the experiment of Medium A the mince was centrifuged and resuspended in a 2 hour incubated unlabeled medium and in Medium B the mince was resuspended in fresh unlabeled medium. These procedures are more fully described under "Experimental."

has been reported to be 6.3 to 6.6 (17), thus, clumping due to the effects of extreme pH appears to be eliminated.

Recent papers have described a lace-like continuum found in the cytoplasm of many cells (18-21). This network has associated with it intensely basophilic small particles of microsomal nature, and appears to account for the basophilic properties of the cytoplasm. The endoplasmic reticulum, as this network has been called, provides a basis for considering the result of this paper and for reconciling them with previous work on the free floating submicroscopic microsomes. Microsomes may normally be a part of, or in association with, this network. In the case of liver and other

tissues, in which free microsome preparations were readily obtained, they could have been dissociated from other structural elements during fractionation. Alternatively, the degree of association between "microsomes" and network may vary for different cells, with hepatic cells representing a type with a greater degree of dissociation. Microscopic examination of the preparations studied in this paper has revealed a peripheralization of basophilic material around adjacent intracellular protein globules rather than an accumulation at their interstices, and is consistent with the association of this basophilic material with a pliable matrix. The size of some of the larger discernible basophilic structures is of the order<sup>1</sup> of  $1\ \mu$ . If these were freely floating bodies, they might not be expected to sediment as easily as has been observed. Their observed sedimentation behavior suggests that they may be strongly associated with some larger easily sedimentable structure such as the endoplasmic reticulum. If the early stages of protein synthesis are associated with such a fixed structure, it might be possible to explain the absence to date of findings of precursor stages such as peptides in the soluble part of the cell.

The relative behavior of the protein fractions discussed here is consistent with the possibility that free amino acids are first incorporated into some part of the cell debris proteins, and that there then occurs a subsequent transfer of labeled precursor substances to the other proteins of the cell. The high activity of the cell debris fraction with respect to the incorporation of amino acids has been observed with all six of the amino acids studied in this work.

Gale and his coworkers at Cambridge (22) have studied a protein fraction obtained after subjecting *Staphylococcus aureus* to sonic vibration. This preparation contains the gross cell structure and is active in incorporating amino acids under conditions favorable for protein synthesis and also under conditions favorable for amino acid exchange. Although their system is different from the one reported here, some analogy may be drawn which might link the structural part of the cell to protein-synthetic reactions.

#### SUMMARY

1 The cytoplasmic ribonucleic acid-containing basophilia and the material of the highest amino acid-incorporating ability in hen oviduct tissue are easily sedimentable.

2 The specific radioactivities of the dicarboxylic amino acids contained in this easily sedimentable fraction are markedly decreased when incubations are continued after the addition of non-isotopic glutamic and aspartic acids, or the replacement of  $C^{14}O_2$  by  $C^{12}O_2$ . The more soluble proteins continue to increase in radioactivity after such dilution.

3 These results are discussed in relation to the endoplasmic reticulum and the mechanism of protein synthesis

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# CHROMATOGRAPHY OF THYROID-STIMULATING HORMONE ON CARBOXYMETHYLCELLULOSE\*

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Evidence has accumulated that bovine thyroid-stimulating hormone (TSH) is a basic protein (2), with a reported sedimentation constant of 10 S,<sup>1</sup> (3, 4). The lack of a standard preparation of the hormone before 1952 makes it difficult to compare the potency of earlier preparations of TSH from different laboratories. Albert (5) summarized the information available prior to 1949 and concluded that the most active preparations, those of Fraenkel-Conrat *et al* (6) and Ciereszko (7), were approximately 100 to 300 times as potent in TSH as the bovine anterior pituitary powder employed as the starting material. Steelman *et al* (2) reported subsequently that TSH could be prepared from beef glands by an electrical transport method and stated that the potency of refractionated material was about 10 U S P units per mg.

In 1953, Heideman (8) described the purification of TSH on the commercial carboxylic acid resin Amberlite IRC-50. Considerable purification was obtained, but the potency of the final preparation was not stated in terms of the U S P reference standard. Subsequently, Crigler and Waugh (9), using IRC-50, reported the preparation of fractions having a potency of 1 to 2 units per mg.

We wish to report a simple method for the further purification of crude preparations of TSH. This is accomplished by chromatography on carboxymethylcellulose, with virtually quantitative recovery during the chromatographic procedure. By this means, in combination with the preliminary fractionation method of Ciereszko (7), about 50 per cent of the TSH present in bovine anterior pituitary powder can be recovered as a product having a potency of at least 5 units per mg, or about 280 times the potency of the starting material.

\* A preliminary account of this work was presented at the meeting of the American Society of Biological Chemists, April, 1956 (1).

<sup>1</sup> White (3) and Fels, Simpson, and Evans (4) have reported a sedimentation constant of 10 S for their preparations of TSH. Recent experiments of Pierce, presented orally at the meeting of the American Society of Biological Chemists, April, 1956, indicate that the sedimentation constant of TSH is actually 2.5 S. A value close to 3.0 S was found, when a preparation of 5 units per mg, described herein, was examined in the ultracentrifuge.

### Methods

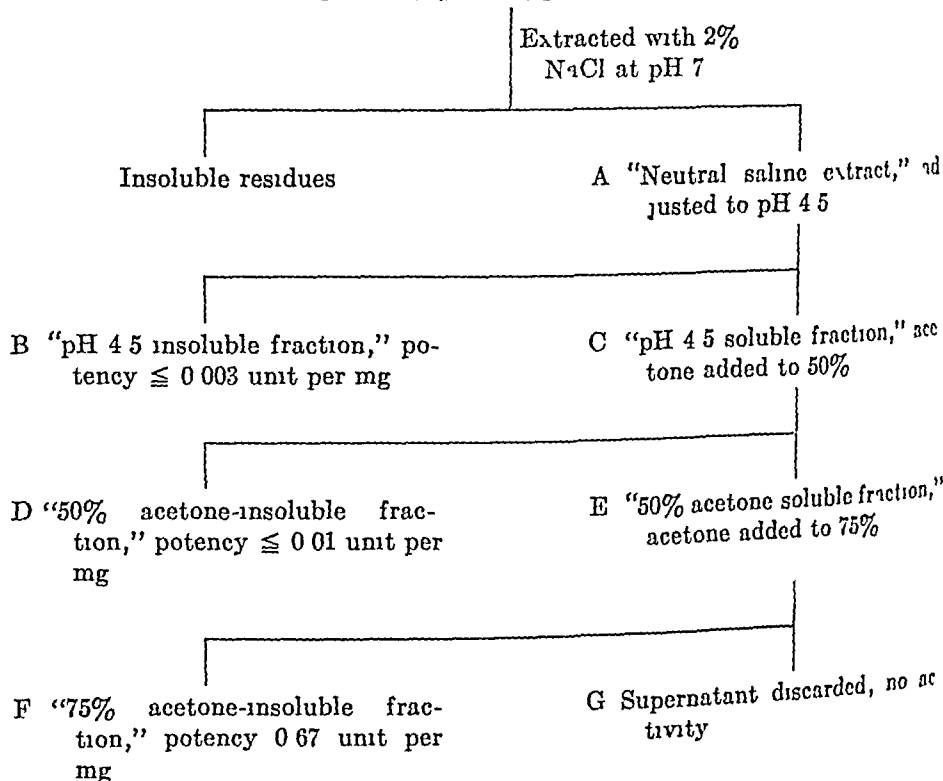
**Chemical Analyses**—Two procedures were used for the estimation of the protein concentration in the various fractions: (1) The absorption at  $276\text{ m}\mu$  was measured in a Beckman model DU spectrophotometer. The TSH preparations studied were found to have ultraviolet absorption values at  $276\text{ m}\mu$  corresponding to an extinction coefficient of 6.5 for a 1 per cent solution. (2) The modified biuret-Folin reaction described by Lowry *et al.* (10) was used. An empirical relationship was established for the various fractions, since the color developed was only proportional to the protein concentration over a short concentration range.

**Bioassays**—Thyroid-stimulating activity was estimated by the depletion of  $\text{I}^{131}$  in the thyroids of day-old chicks, according to the procedure of Bates and Cornfield (11). The accuracy is about  $\pm 30$  per cent for individual fractions from a column and  $\pm 20$  per cent for pooled, lyophilized fractions. All potencies are expressed in terms of U. S. P. units of TSH.

#### DIAGRAM 1

##### *Fractionation of Anterior Pituitary Powder for TSH\**

Bovine anterior pituitary powder, potency = 0.017 unit per mg



\* All steps were carried out at  $2-4^\circ$

*Preparation of Crude TSH*—Frozen bovine pituitaries were dissected and the anterior lobes dried by lyophilization. The lyophilized glands were ground in a Wiley mill, a 40 mesh screen being used. This anterior lobe powder was extracted at 2° with 2 per cent NaCl, adjusted to pH 7 with NaOH, and fractionated according to the scheme outlined in Diagram 1, which is similar to that of Ciereszko (7). Approximately 60 to 70 per cent of the activity was concentrated in Fraction F, different batches of which had a potency of 0.67 to 1 unit per mg and thus were 40 to 60 times as potent as the original powder. Fraction F contained no prolactin and less than 0.1 U.S.P. unit of adrenocorticotropin per mg.

*Desalting of TSH Solutions*—While no significant loss of TSH potency was observed during dialysis, desalting with a mixed bed ion exchanger proved to be simple and less time-consuming. A commercial mixed bed resin MB-3, containing an indicator, was purchased ready for use.<sup>2</sup> The pH of the desalted TSH solutions was usually about 8, and a faint turbidity was sometimes observed, suggesting that the isoelectric TSH has a limited solubility in salt-free solution. Raising or lowering the pH or the addition of a small amount of salt caused this turbidity to disappear. No significant loss of TSH activity was observed during this procedure.

#### RESULTS AND DISCUSSION

*Experiments with IRC-50*—On the basis of the report by Heideman (8) that TSH could be purified with IRC-50 and by Steelman *et al.* (2) that the isoelectric point of the hormone was at about pH 8, chromatographic experiments with TSH on IRC-50 columns were made. The resin was obtained as a 200 mesh powder of IRC-50 (XE-64)<sup>2</sup> and was recycled according to the procedure of Hirs, Moore, and Stein (12).

The first set of conditions employed was that of placing the TSH on a column which had been equilibrated at pH 6.0 with 0.20 M sodium phosphate buffer. In a typical experiment shown in Fig. 1 (also Experiment 1, Table I), the TSH was retained on the column, while about 35 per cent of the protein, which contained less than 1 per cent of the activity placed on the column, emerged between Fractions 16 and 64. Fractions of 0.5 ml were collected and the experiment was carried out at 25°. The solvent was changed to 1.0 M NaCl at Fraction 170 and the TSH was eluted between Fractions 200 and 208. The highest potency was estimated to be about 5 units per mg at Fraction 203. The recovery of TSH was only 50 per cent of the original activity placed on the column. When the fractions containing TSH were pooled, desalted, and lyophilized, the potency in this and in other experiments was between 2 and 4 units per mg, as shown in

<sup>2</sup> These resins were obtained from the Rohm and Haas Company, Philadelphia 5, Pennsylvania.

Table I, in which the yields obtained in a number of experiments are summarized

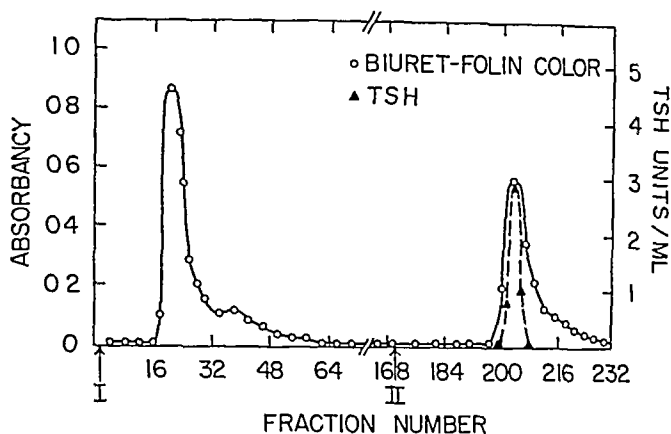


FIG 1 Chromatogram of crude TSH (Fraction F, Diagram 1) on IRC-50 80 mg (1 U S P unit per mg) on a 0.9 × 30 cm XE-64 column. Effluent collected in 0.5 ml fractions. Solvents I, 0.20 M sodium phosphate, pH 6.0, II, 1.0 M NaCl. Temperature, 25°. O, biuret-Folin color, ▲, TSH activity in U S P units per ml of effluent.

TABLE I  
Recovery of TSH from IRC-50 Columns (XE-64)\*

Experiment No	Column size	Starting material		TSH eluted		
		Potency	Total TSH	Potency of pooled peak	Total TSH	Yield
	cm	U S P unit per mg	U S P units	U S P units per mg	U S P units	per cent
1	0.9 × 30	1.0	8.5	2.0†	4.0	47
2	0.9 × 30	1.0	12.0	4.0†	6.0	50
3‡	0.9 × 20	0.2	2.5	3.3‡	1.3	52
4	1.9 × 30	1.0	190	2.0†	116	61
5‡	1.9 × 30	0.2	400	3.3§	250	63

\* TSH was adsorbed at pH 6 from 0.2 M sodium phosphate buffer and was eluted with 1 M NaCl.

† Estimated from the bioassay results relative to the ultraviolet absorption of effluent at 276 mμ or the biuret-Folin color.

‡ The starting material was a growth hormone preparation.

§ Estimated by assay of lyophilized, pooled fractions.

Other conditions of adsorption and elution were also studied. It was found, for instance, that TSH was not retained on columns at pH 7.0 in 0.20 M sodium phosphate buffer. Various elution procedures including gradients of increasing sodium ion concentration and pH failed to give additional purification or better recovery than those indicated in Table I.

These results are consistent with the observations of Pierce and Nyc (13), who found that TSH is adsorbed on IRC-50 in 0.20 M sodium phosphate at pH 5.9, but passes through such a column at pH 6.4.

*Experiments with Carboxymethylcellulose*—Carboxymethylcellulose (CM-W) was prepared from standard grade Whatman cellulose powder by the method of Peterson and Sober (14). In preliminary equilibration experiments carried out in test tubes, it was found that 1 mg of CM-W would adsorb about 1 unit of TSH from solution at pH 6.0 in 0.01 M sodium phosphate buffer. Little or no adsorption occurred at pH 8.5 or above. Accordingly, a 1.9 × 19 cm column containing about 8 gm of CM-W

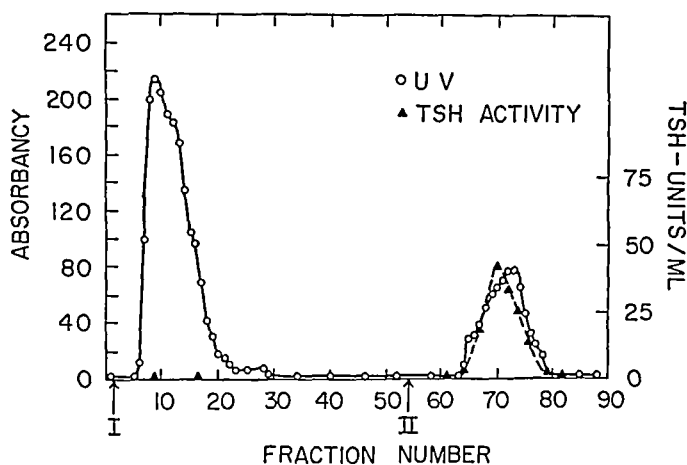


FIG 2 Chromatogram of crude TSH (Fraction F, Diagram 1) on CM-W 10 gm (0.7 U S P unit per mg) on a 1.9 × 19 cm CM-W column. Effluent collected in 1 ml fractions. Solvents I, 0.01 M sodium phosphate, pH 6.0, II, 1.0 M NaCl. Temperature, 25°. ○, ultraviolet absorption at 276 mμ, ▲, TSH activity in U S P units per ml of effluent.

was prepared and equilibrated at pH 6.0 with a 0.01 M sodium phosphate buffer. 1 gm of a TSH preparation, which had a potency of 0.7 unit per mg, was dissolved in 2 ml of buffer and placed on the column. Fractions of 1 ml were collected at the rate of three to four fractions an hour. The experiment was carried out at 25°. After 53 fractions had been collected, the solvent was changed to 1.0 M NaCl, and an additional thirty-five fractions were collected. The effluent fractions were diluted with 2 ml of water, and their ultraviolet absorption was measured at 276 mμ. Bioassays were performed at appropriate intervals. As shown in Fig 2 (also Experiment 3, Table II), approximately 70 per cent of the protein was accounted for in Fractions 5 to 30. These fractions contained less than 0.1 per cent of the TSH placed on the column. Of the 700 units in the starting material, 718 were accounted for in the elution peak between Fractions 65 and 80. When Fractions 67 to 80 were pooled, desalted on a



mixed bed resin column, and lyophilized, 240 mg of powder were recovered which had a potency of 3 units per mg. There was therefore no loss of

TABLE II  
*Recovery of TSH from Carboxymethylcellulose (CM-W) Columns*

Experiment No	Column size	Starting material		TSH eluted		
		Potency	Total TSH	Potency of pooled peak	Total TSH	Yield

Stepwise elution\*

	cm	U S P units per mg	U S P units	U S P units per mg	U S P units	per cent
1	0.9 × 25	0.7	20	2.0†	17	85
2	0.9 × 12	0.7	11	3.0†	12	100
3	1.9 × 19	0.7	700	3.0‡	718	100
4	0.9 × 12	3.0	19	3.0†	17	80
5§	0.9 × 12	0.6	18	3.6†	17	95

Gradient elution

6	0.9 × 18	0.7	48	2.5†	40	83
7	0.9 × 12	3.0	36	5.0†	29	81
8	0.9 × 20	1.5	42	8.0†	20	48
				2.8†	18	43
9	0.9 × 22	1.5	198	5.0‡	140	73
				1.4‡	28	14
10	0.9 × 30	0.6	1700	3.0‡	1700	70
11	0.9 × 15	5.0	50	6.0†	35	70
12	0.9 × 20	1.75	700	3.5‡	819	117

\* Adsorption at pH 6.0, 0.01 M sodium phosphate, elution with 1.0 M NaCl, except where indicated otherwise.

† Estimated from the bioassay results relative to the ultraviolet absorption of effluent at 276 mμ.

‡ Estimated by assay of lyophilized, pooled fractions.

§ Adsorption at pH 7.0, 0.01 M sodium phosphate, elution with 0.2 M Na HPO<sub>4</sub>.

|| TSH peak recovered in two fractions.

potency during the desalting operation, and the biological activity was recovered quantitatively.

As can be seen in a comparison of Figs 1 and 2, there is a qualitative similarity between the elution patterns for IRC-50 and CM-W. However, a comparison of the recovery data in Table I with that in Table II shows that the yield of purified TSH from CM-W is nearly quantitative in contrast to the 50 per cent yield from IRC-50.

Since the potency was not found to be uniform across the TSH elution peak in Fig 2, or in similar stepwise elution experiments listed in Table II, it seemed likely that further purification could be achieved. Attempts to find conditions under which equilibrium chromatography could be carried out in a manner similar to that performed by Hirs, Moore, and Stem with ribonuclease (12) were unsuccessful. It was found that at pH 7.0 in 0.20 M sodium phosphate buffer, the TSH was not adsorbed on CM-W columns, while at pH 7.0 in 0.10 M sodium phosphate, the activity was spread over a great many fractions with no apparent purification being achieved. Several gradient elution schedules were then tried, and it was

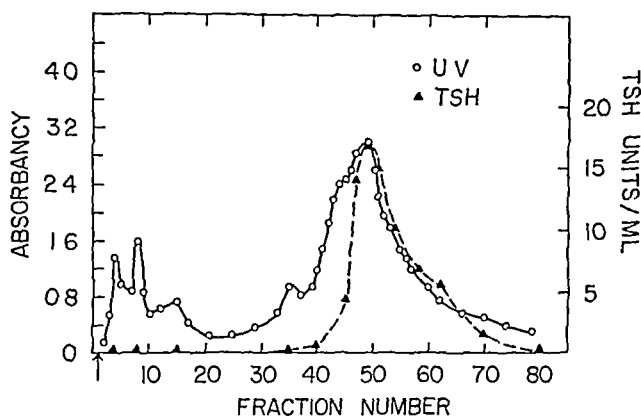


Fig 3 Chromatography of partially purified TSH on CM-W 132 mg (15 U S P units per mg) on a  $0.9 \times 22$  cm column. Effluent collected in 1 ml fractions. Temperature,  $2^{\circ}$  ↑, gradient from 0.01 M sodium phosphate, pH 6.0, rising to 1.0 M NaCl. Mixing chamber volume, 100 ml, ○, ultraviolet absorption at  $276 m\mu$ , ▲, TSH activity in U S P units per ml of effluent.

found that quite steep gradients were best for the elution of TSH, rising towards a sodium ion concentration of 1.0 M within 10 retention volumes. Again, a gradient which only reached a concentration of 0.5 M sodium ion when 10 retention volumes had passed through the column was unsuccessful in eluting the TSH quantitatively or with any significant degree of purification. However, when the gradient was sufficiently steep, the TSH emerged at a sodium ion concentration of 0.3 M to 0.4 M.

In a typical experiment, Fig 3 (also Experiment 9, Table II), 132 mg of a preparation having a potency of 15 units per mg were placed on a  $0.9 \times 22$  cm column at pH 6 in 0.01 M sodium phosphate. A gradient to 1.0 M NaCl was begun at Fraction 3. The mixing chamber contained 100 ml of the 0.01 M sodium phosphate buffer. Fractions of 1 ml were collected at a rate of four fractions an hour at the start of the experiment, which was carried out at  $2^{\circ}$ . Approximately 200 units of activity were

accounted for in Fractions 40 to 70, and the highest potency in Fraction 51 was estimated to be at least 5 units per mg. Fractions 49 to 65, which

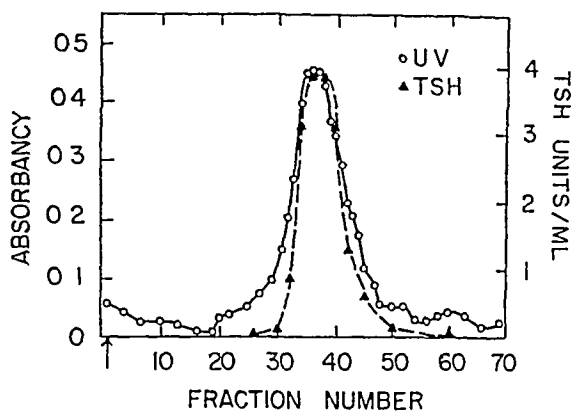


FIG 4 Rechromatography of purified TSH on CM-W 10 mg ( $5.0 \pm 1.0$  U S P units per mg) on a  $0.9 \times 15$  cm column. Effluent collected in 1 ml fractions. Temperature,  $2^\circ$  ↑, gradient from 0.01 M sodium phosphate, pH 6.0, rising to 1.0 M NaCl. Mixing chamber volume, 100 ml, ○, ultraviolet absorption at 276 mμ, ▲, TSH activity in U S P units per ml of effluent.

TABLE III  
Recovery of Bovine TSH

Fraction	Dry weight	TSH potency	Total TSH	Fraction of original activity
	gm	U S P units per mg	U S P units	per cent
Bovine anterior pituitary powder	100	0.017	1700	100
Insoluble, pH 4.5	20	0.003	60	4
50% acetone-insoluble	8.99	0.01	90	5
75% " "	1.58	0.7	1190	70
1st CM-W column				
Unadsorbed	1.03	0.03	33	2
NaCl eluate	0.379	3.0	1140	67
2nd CM-W column,* gradient elution				
Fraction a	0.05	0.01	0.5	0.1
" b	0.162	$5.0 \pm 1.0^\dagger$	810	48
" c	0.114	$1.4 \pm 0.4^\dagger$	160	9

\* Fraction a corresponds to the unadsorbed fractions in stepwise elution experiments.

† 95 per cent confidence limits.

assayed uniformly between 4 and 6 units per mg, were pooled, desalted, and lyophilized. 28 mg of lyophilized powder were recovered. The potency was found to be  $5.0 \pm 1$  units per mg. An additional 20 mg of material, which contained 1.4 units per mg, were recovered in

Fractions 45 to 48 and 65 to 74 The recovery of TSH was thus about 170 units or between 80 and 90 per cent of the activity placed on the column

When 10 mg of the material having a potency of 5 units per mg were rechromatographed on a  $0.9 \times 15$  cm column, the gradient elution pattern shown in Fig 4 (also Experiment 11, Table II) was obtained in which a uniformly potent peak emerged between Fractions 32 and 45 Approximately 70 per cent of the TSH was accounted for in these fractions which had a potency of 5 to 7 units per mg

In terms of the original anterior pituitary powder, the purification obtained by the combination of fractionation with acetone, followed by chromatography on carboxymethylcellulose, is about 300 times, and the over-all yield of highly potent TSH is about 50 per cent as shown in Table III From 100 gm of anterior pituitary powder having a potency of 0.017 unit per mg, one can obtain 162 mg of TSH having a potency of 5 units per mg

#### SUMMARY

The chromatographic behavior of thyroid-stimulating hormone (TSH) on IRC-50 and on carboxymethylcellulose columns was studied under various conditions The same degree of purification was achieved in both cases, but the recovery of TSH from carboxymethylcellulose columns was nearly quantitative in contrast to only a 50 per cent yield from IRC-50 columns

TSH having a potency of 5 to 7 U S P units per mg was obtained from bovine pituitary powder by acetone fractionation and subsequent chromatography on carboxymethylcellulose

We wish to thank Miss Alice Laskey and Mr Tulane Howard for their able assistance We are indebted to Dr Elbert A Peterson and Dr Herbert A Sober for their cooperation and advice

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# THE DEGRADATION OF HEPARIN BY BACTERIAL ENZYMES

## I ADAPTATION AND LYOPHILIZED CELLS\*

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The physiological role of heparin has been the subject of considerable research in recent years because of both its anticoagulant property and its ability to induce the appearance of lipoprotein lipase in the circulation. Yet, the structural details of this mucopolysaccharide are still essentially unknown. Work in several laboratories (2-4) has resulted in the general conclusion that heparin is composed of equimolar amounts of glucosamine and glucuronic acid with one to three sulfate moieties per disaccharide unit. One of the sulfates is bound in an amide linkage to the amino group of glucosamine (5), the others are esterified on the sugars at unknown positions. The positions of the glycosidic linkages are also unknown.

Heparin may be hydrolyzed with acid only under rather rigorous conditions which result in considerable caramelization, especially of the glucuronic acid, and extensive splitting. Thus, the products are mono- or, at the most, disaccharides from which all of the sulfate groups have been removed (6). An analysis of these products does not permit one to postulate a unique structure for the parent molecule. Enzymatic hydrolysis, however, should make possible the quantitative isolation of partial hydrolysis products, the characterization of which would reveal the fine structure of heparin. In addition, these products of partial enzymatic hydrolysis might retain biological activity.

This paper describes the isolation of a soil bacterium which is able to use heparin as its sole source of carbon, nitrogen, and sulfur, some experiments on the adaptation of the bacteria to heparin, and the characteristics of the degradation of heparin by lyophilized cells. The degradation of heparin by cell-free extracts is described in Paper II.

## EXPERIMENTAL

**Methods**—The disappearance of heparin was routinely followed by the decrease in metachromasia with azure A (7) as measured at 490 mμ in a

\* A preliminary report of some of the material in this paper has been published (1).

† Participant in the Foreign Research Scientist Program of the International Cooperation Administration.

**Coleman junior spectrophotometer** This method is quite reproducible, but little information as to the nature of the degradative process is obtained. A decrease in metachromasia would result from a loss of sulfate groups or extreme shortening of the polysaccharide chain. Reducing groups were determined by the method of Fohn and Malmros (8), an equimolar mixture of glucuronic acid and glucosamine was used as a standard. Periodate-oxidizable groups were determined by the spectrophotometric method of Dixon and Lipkin (9) and amino sugars by the Elson-Morgan reaction (10) with glucosamine as the standard. It should be recognized that, in the absence of the proper standards, the molar values reported must be considered only approximate. In every instance, except when noted, the rates of decrease in metachromasia and increase in reducing groups were parallel.

**Isolation of *Bacterium***—The bacterium was isolated from dry soil by enrichment culturing at 24° on a liquid medium which contained 0.1 per cent sodium heparin, 0.1 per cent ammonium sulfate, 0.1 M phosphate buffer, pH 7.4, and trace amounts of magnesium, calcium, ferrous, manganous, and molybdate ions and on silica gel plates with this medium evaporated on the surface. The bacterium is a gram-negative, non-spore-forming, very small, motile rod identified as a *Flavobacterium*, hereinafter referred to as *Flavobacterium heparinum*. It is an obligate aerobe which grows optimally at 24° and not at all at 0° or 37°. The optimal pH for growth is 6.5 to 7.0.

Many bacteria were isolated during the enrichment culture procedure which were able to grow weakly on the above medium although they were unable to degrade heparin. This was undoubtedly due to the presence of small quantities of impurities in the commercial heparin. The heparin was progressively "purified" by being used as substrate for the non-heparin utilizing bacteria with obvious advantage in the subsequent enrichment culturing. It was essential, therefore, during the isolation procedure to follow the disappearance of heparin and not growth.

## RESULTS AND DISCUSSION

### *Adaptation*

Although *F. heparinum* grows well on heparin alone, it is not economically feasible to use it as the sole carbon source in large scale cultures. The bacteria were routinely grown on a medium which contained 0.35 per cent trypticase, 0.06 per cent phytone, 0.05 per cent glucose, 0.002 per cent sodium heparin, 0.1 per cent NaCl, and 0.05 per cent  $K_2HPO_4$ . The medium was adjusted to pH 6.5 with sulfuric acid. Cells grown on this medium were unable to degrade heparin. They could be adapted to heparin, however, by the following procedure. Cells were harvested after growth for 36 hours, washed with phosphate buffer (0.025 M, pH 8.0), and then su-

pended in a volume of buffer equal to one-tenth the volume of the original growth medium. Casein hydrolysate (final concentration, 0.05 per cent) and sodium heparin (final concentration, 0.1 per cent) were added and the suspension was aerated vigorously at 24°. Adaptation was followed by the disappearance of heparin and was considered complete when the rate of disappearance became constant. This usually occurred between 2 and 4 hours after the onset of aeration when approximately two-thirds of the heparin had been degraded. Although it is theoretically possible for adap-

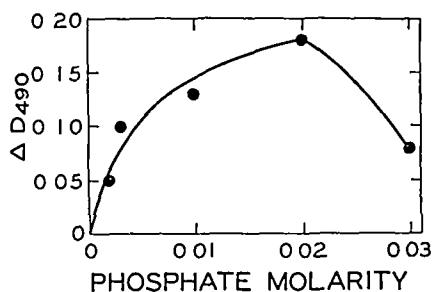


FIG 1

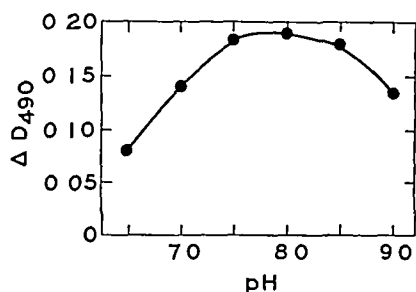


FIG 2

FIG 1 Dependence of the rate of adaptation on the concentration of phosphate. Cells were harvested in the usual manner, washed with 0.02 M arsenate buffer, pH 8, and suspended in 35 ml of arsenate buffer. 0.5 ml of 10 per cent casein hydrolysate and 50 mg of sodium heparin were added. The suspension was divided into 3.5 ml portions and increasing amounts of 1 M phosphate buffer, pH 8, were added to the different tubes. The final volume was adjusted to 5.0 ml with the arsenate buffer and the tubes were aerated at room temperature. Aliquots of 0.02 ml were removed at various time intervals for the determination of metachromasia. The values plotted are those obtained 3.5 hours after the onset of aeration, at the optimal phosphate molarity two-thirds of the heparin had been degraded at this time.

FIG 2 Influence of pH upon the rate of adaptation. Adaptation was carried out as described in the text with phosphate buffers at the indicated pH values. The values reported are those obtained after 3 hours of adaptation.

tation to occur under conditions which would not permit degradation of heparin, it was found that in all instances in which adaptation was not detected by the above procedure lyophilized cells were unable to degrade heparin under optimal conditions.

**Phosphate Requirement**—Adaptation did not occur in the absence of casein hydrolysate, aeration, or phosphate, or at 0° or 37°. The absolute requirement for phosphate is demonstrated in Fig 1. No other ion has been found which will replace it. Above 0.03 M, all salts inhibited adaptation. This inhibition was complete at 0.1 M.

**pH**—The data summarized in Fig 2 indicate that adaptation can occur over a wide pH range with an optimal rate between pH 7.5 and 8.5.

**Influence of Heparin in Growth Medium**—Cells grown in the presence of a



low concentration of heparin, although completely unable to degrade heparin, are able to adapt to heparin much more rapidly than cells not previously exposed to heparin (Fig 3)

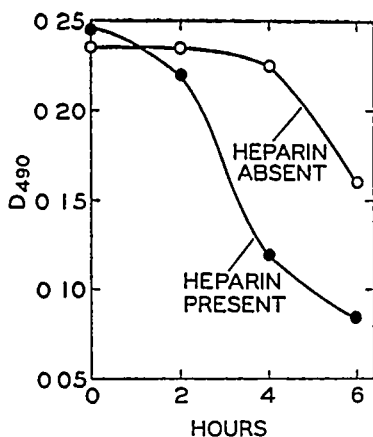


FIG 3 Effect of heparin in the original growth medium upon the subsequent rate of adaptation. Adaptation was performed as described in the text. The only difference was the presence or absence of heparin in the original growth medium.

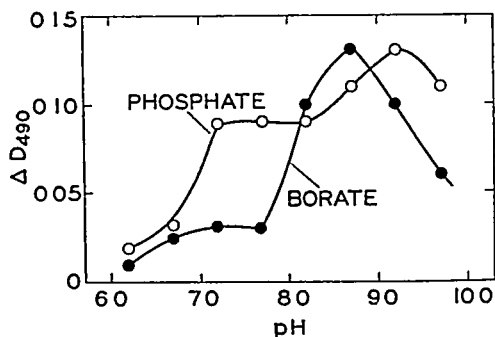


FIG 4

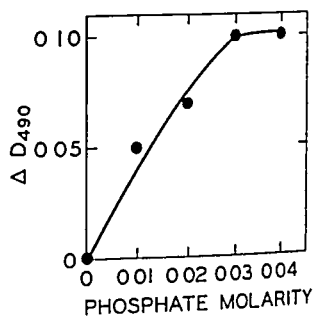


FIG 5

FIG 4 Comparison of the degradation of heparin by lyophilized cells in phosphate and borate buffers. 300 mg of lyophilized cells were suspended in 20 ml of water. 2.5 ml of this suspension, 2 ml of 0.1 M phosphate or borate buffer, and 0.5 ml of 1 per cent sodium heparin were mixed and incubated at room temperature. At 0, 50, and 100 minutes, 0.02 ml aliquots were removed and tested for metachromasia. The values plotted are those obtained after incubation for 100 minutes. The final molarity (0.04 M) was too high for optimal activity but was necessary for adequate buffering. Even so, at the optimal pH, one-half the heparin had been degraded in 100 minutes.

FIG 5 Phosphate requirement for the degradation of heparin by lyophilized cells at pH 7.2. 300 mg of cells were suspended in 20 ml of distilled water. 2.5 ml of this suspension were diluted to a total volume of 5 ml which contained 5 mg of sodium heparin and a mixture of phosphate and arsenate buffers (0.04 M). The molarity of the phosphate buffer was as plotted. The values plotted are those obtained after incubation for 150 minutes at room temperature.

*Lyophilized Cells*

Bacteria grown and adapted as described above were collected, washed once with phosphate buffer (0.025 M, pH 8.0), and washed twice with water at 0°. They were then lyophilized and stored in a vacuum desiccator at -15°.

*Degradation of Heparin in Phosphate and Borate Buffers*—When incubated in phosphate buffer, lyophilized cells degraded heparin over a broad range of pH (Fig. 4). In borate buffer, however, a much sharper peak was obtained. The difference between the rate of degradation of heparin in the

TABLE I  
*Reactions Involved in Degradation of Heparin by Lyophilized Cells*

pH	Buffer	Metachromasia decrease	Reducing group increase	Amino sugar increase	Periodate consumption increase
		$\Delta D_{490}$	$\mu\text{moles}$	$\mu\text{moles}$	$\mu\text{moles}$
7.2	Phosphate	0.07	3.6	1.5	42
	Borate	0	2.7	0.4	0
9.2	Phosphate	0.12	4.8	2.4	42
	Borate	0.10	3.6	1.5	15

300 mg. of lyophilized, adapted cells were suspended in 15 ml. of distilled water. Each vessel contained 3 ml. of the cell suspension and 6 mg. of sodium heparin in a total volume of 6 ml. of the indicated buffer (0.04 M). The vessels were incubated at 24° and aliquots were removed at 0, 50, and 100 minutes for determination of metachromasia (0.02 ml.), reducing groups (0.2 ml.), amino sugars (0.2 ml.), and periodate consumption (0.2 ml.). The micromole values are calculated for the total volume of 6 ml. All values reported are those obtained at 100 minutes.

two buffers was most pronounced from pH 7 to 8. Further, it could be demonstrated (Fig. 5) that a direct proportionality exists between phosphate concentration and the rate of heparin degradation at pH 7.2. Thus there appear to be two pathways for the degradation of heparin in lyophilized cells: one which requires phosphate and has an optimal pH around 7.5 and one which has an optimal pH of 8.5 to 9.0 and has no phosphate requirement.<sup>1</sup>

*Reactions Involved in Heparin Degradation*—The degradation of heparin by lyophilized cells might be expected to be the result of the action of several enzymes. Following the disappearance of heparin by the loss of metachromatic activity does not provide any information as to the nature of the enzymatic reactions involved. Therefore, an experiment was carried out in which the disappearance of heparin and the appearance of

<sup>1</sup> An earlier statement (1) that the reaction which requires phosphate also requires  $\text{Mg}^{2+}$  was incorrect.

reducing groups, amino sugar, and periodate-titratable groups were simultaneously determined. The results are presented in Table I.

In phosphate buffer at pH 7.2 and 9.2 and in borate buffer at pH 9.2, an increase in reducing groups, amino sugar, and periodate-titratable groups, as well as a decrease in metachromasia, was observed. The first two observations indicate the presence of a glycosidase and a sulfamidase in the lyophilized cells. Since the ratio of the increase in periodate consumed to reducing groups formed was much too high to be accounted for by the activities of the glycosidase and sulfamidase alone, the lyophilized cells apparently contain a sulfesterase.

In borate buffer at pH 7.2, the only significant change was an increase in the reducing groups.

*Activity of Lyophilized Cells of Unadapted Bacteria*—Lyophilized cells of unadapted bacteria were completely unable to metabolize heparin as determined by all of the above methods.

#### SUMMARY

*Flavobacterium heparinum*, a bacterium which is able to utilize heparin as its sole source of carbon, nitrogen, and sulfur, has been isolated from soil by enrichment culturing. A procedure has been described whereby bacteria grown on a non-heparin medium can be adapted to heparin. The degradation of heparin by lyophilized, adapted cells has been studied.

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# THE DEGRADATION OF HEPARIN BY BACTERIAL ENZYMES

## II ACETONE POWDER EXTRACTS\*

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In the accompanying paper (2) the isolation and adaptation of *Flavobacterium heparinum* and experiments on the degradation of heparin by lyophilized, adapted bacteria are described. The isolation of this organism was originally undertaken with the ultimate goal of obtaining purified enzymes which could be used as analytical tools for a study of the structure of heparin and for the preparation of subunits which might possess biological activity. To this end, the degradation of heparin in cell-free extracts of the adapted bacteria has been investigated.

### EXPERIMENTAL

**Materials**—Sodium heparin (120 units per mg) was a product of The Upjohn Company. It had an S/N ratio of 2/1 and upon acid hydrolysis 90 per cent of the theoretical glucosamine content was recovered (ninhydrin reaction). The heparin gave no ninhydrin reaction, consumed no periodate, and contained essentially no free reducing groups.

Hyaluronic acid, chondroitin sulfate, pectin, pectic acid, polygalacturonic acid, and chitin sulfate were all commercial products.

**Methods**—The analytical methods were identical to those described previously (2). Glucosamine was used as the standard for the amino sugar determinations and an equimolar mixture of glucosamine and gluconic acid for the reducing group experiments. Metachromasia was determined on 0.02 ml aliquots of the reaction mixture.

**Acetone Powder Extract**—The bacteria were grown and adapted as described previously (2). Adapted cells were collected, washed with water, blended with a large volume of acetone ( $-20^{\circ}$ ), filtered, and blended again with acetone and the final filter cake was air-dried. The acetone powder is stable indefinitely when stored in a vacuum desiccator at  $-15^{\circ}$ . Unless otherwise stated, the powder was extracted with phosphate buffer (0.025 M,

\* A preliminary report of some of the material in this paper has been published (1).

† Participant in the Foreign Research Scientist Program of the International Cooperation Administration.

pH 8.0, 10 mg per ml) for 1 hour at 0°, the insoluble residue was removed by centrifugation, and the resultant solution was used. The final pH of the extract was 7.5 and it had a protein concentration of about 1.5 mg per ml.

### Results

*Enzymatic Activity of Extract*—Heparin was incubated with the acetone powder extract and the decrease in metachromasia and the increase in reducing groups, periodate-titratable groups, and amino sugar (Elson-Morgan reaction) followed simultaneously. The values obtained for all four determinations were directly proportional to the time of incubation (Fig. 1). Further, it was demonstrated that the rates of decrease in metachromasia

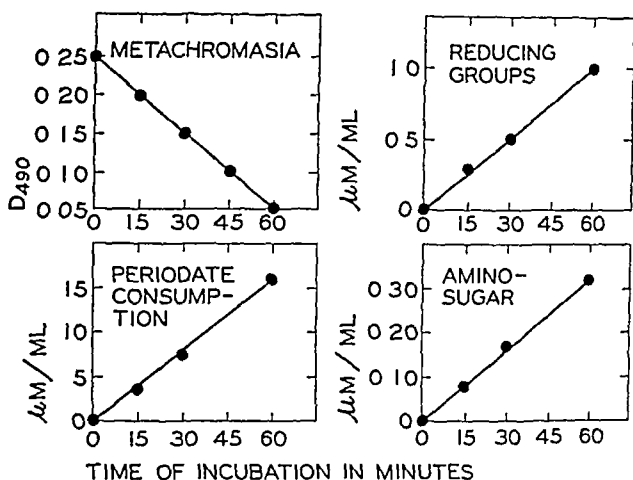


FIG. 1. The enzymatic reactions involved in the degradation of heparin by acetone powder extracts. The incubation vessel contained 6 ml. of the acetone powder extract and 6 mg. of sodium heparin. Incubation temperature, 24°.

and increase in reducing groups were directly proportional to the concentration of extract used (Fig. 2) and that the rate of reaction was also proportional to the concentration of substrate (Fig. 3). The enzymatic activity of the extract was completely stable to prolonged dialysis against distilled water and completely inactivated by heating at 40° for 5 minutes.

The positive Elson-Morgan reaction required the addition of acetone and the resultant pink color completely disappeared at alkaline pH but reappeared upon the addition of acid. This indicates (3) that the reactant is indeed an amino sugar and not an amino acid plus a reducing sugar.

No enzymatic activity was detectable by any of the above determinations in the absence of heparin nor when extracts of acetone powders of unadapted bacteria were used.

*Effect of pH*—The hydrolysis of heparin, as measured by the increase in

reducing groups as well as the decrease in metachromasia, occurred optimally at pH 7.0 to 7.5 (Fig. 4). The reaction in lyophilized cells (2), which proceeds maximally at pH 9.0, was absent from the extracts

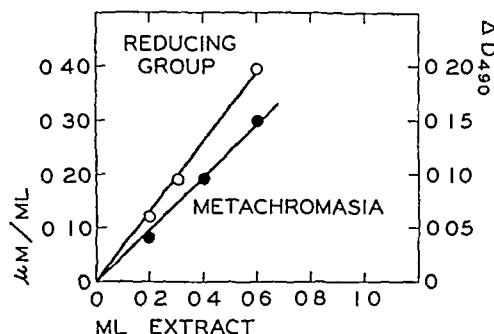


FIG. 2

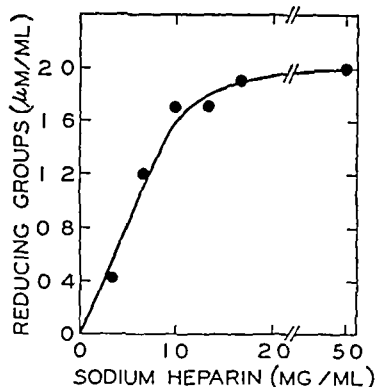


FIG. 3

FIG. 2 Proportionality between the rate of degradation of heparin and the concentration of extract. Each vessel contained 1 mg of sodium heparin and the indicated volume of extract in a total volume of 1 ml of phosphate buffer (0.025 M, pH 7.5). In this experiment the extract contained 4.5 mg of protein per ml. The vessels were incubated for 30 minutes at 24°.

FIG. 3 Proportionality between the rate of degradation of heparin and concentration of substrate. Each vessel contained the indicated amount of sodium heparin per ml of acetone powder extract. The vessels were incubated at 24° for 100 minutes.

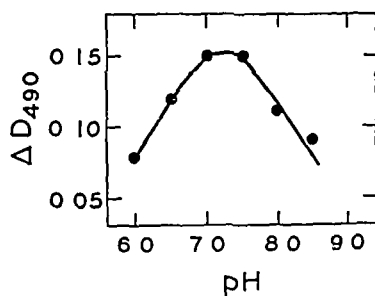


FIG. 4 Degradation of heparin as a function of pH. The extract was prepared in the usual manner and dialyzed against water overnight. The resultant precipitate was suspended homogeneously and equal aliquots were made up to the original volume in 0.025 M phosphate buffer at the indicated pH values. 1 ml of enzyme was incubated with 1 mg of sodium heparin for 1 hour at 24°.

*Requirement for Ions*—In some preliminary experiments it was noted that extracts prepared with glycylglycine buffer were much less active than those prepared with phosphate buffer. To investigate this matter further, the acetone powder was extracted with phosphate buffer in the usual man-

ner and the extract was dialyzed overnight against three changes of distilled water. After the dialysis the precipitated protein was redissolved in glycylglycine buffer (0.025 M, pH 7.5) and assayed with and without the addition of 0.025 M phosphate buffer, pH 7.5 (Table I).

TABLE I  
*Ion Requirement for Degradation of Heparin*

	Glycylglycine	Glycylglycine + phosphate
Metachromasia, $\Delta D_{490}$	0	0
Reducing groups, $\mu\text{mole per ml}$	0.06	0.50
Periodate consumption, $\mu\text{moles per ml}$	0	8.1
Amino sugar, $\mu\text{mole per ml}$	0.07	0.23

The vessels were incubated for 30 minutes at 24°. For other details see the text.

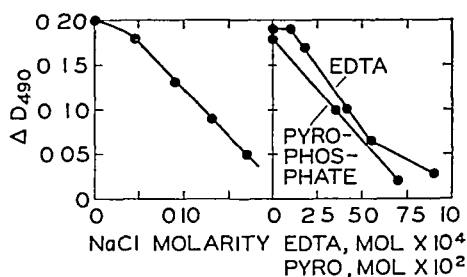


FIG 5

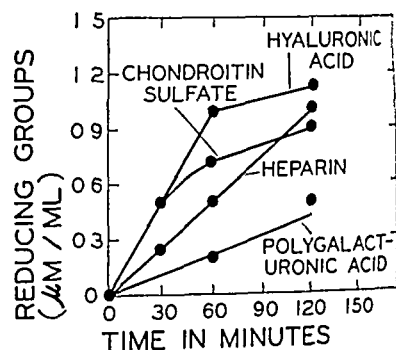


FIG 6

FIG 5 Inhibition of the degradation of heparin by NaCl, EDTA, and pyrophosphate. The activity was measured in the usual manner with 1 ml of extract incubated with 1 mg of sodium heparin in the presence of NaCl, EDTA, or pyrophosphate at the indicated molarities. The vessels were incubated at 24° for 1 hour.

FIG 6 Hydrolysis of other mucopolysaccharides. 2 ml of the acetone powder extract were incubated with 2 mg of each substrate at 24°.

Such preparations were completely inactive without the addition of ions, but there appeared to be almost no specificity with respect to either the cation or anion. Sodium chloride, sodium sulfate, potassium chloride, magnesium chloride, ammonium sulfate, sodium arsenate, and sodium citrate could all replace the potassium phosphate. The optimal molarity was approximately 0.01. It is of special interest, however, to note that sodium borate was unable to activate the system, since heparin is not degraded by lyophilized cells suspended in borate buffer at pH 7.5.

*Inhibition by Ions*—The degradation of heparin was inhibited by any salt at a relatively low molarity. The results of a typical experiment in

which NaCl was used are shown in Fig 5. Pyrophosphate and ethylenediaminetetraacetate (EDTA) ions inhibited at significantly lower molarities. The inhibition by EDTA does not appear to be due to chelation since the extract was reactivated by dialysis.

*Substrate Specificity*—The acetone powder extract has been assayed for its ability to degrade other mucopolysaccharides (Fig 6). Hyaluronic acid and chondroitin sulfate were hydrolyzed at a more rapid rate than heparin. Pectic acid and polygalacturonic acid were hydrolyzed at about one-third to one-half the rate of heparin, while pectin and chitin sulfate were hydrolyzed only very slowly.

*Biological Activity of Degraded Heparin*—Heparin was incubated with the extract (1 mg of heparin per ml) for 1 hour at 24° and the reaction was stopped by placing the vessel in a boiling water bath for 10 minutes. The slight turbidity which formed was removed by centrifugation and the supernatant solution was assayed for anticoagulant activity and the ability to induce the appearance of lipoprotein lipase in the blood. It was completely inactive. Controls, in which the extract was boiled immediately after the addition of the heparin or extracts of unadapted cells were used, retained complete activity.

#### DISCUSSION

Jaques and his coworkers (4-6) have described a preparation from mammalian liver which is said to possess "heparinase" activity. This activity, however, is extremely low and no evidence has been provided as to the nature of the enzymatic process involved. With this exception, the bacterial extract described in this paper is the only known enzyme system capable of catalyzing the degradation of heparin.

It is apparent from the data reported that several enzymes are responsible for the "heparinase" activity of the acetone powder extract. The formation of amino sugar from heparin demonstrates the presence of a glycosidase able to split the glucosaminidic bond as well as a sulfamidase, since a positive Elson-Morgan reaction requires that both the aldehyde and amino groups be free. The sulfamidase is the first enzyme of this type to be found.

The observed reducing group values were 3 times as high as those for the amino sugar when glucosamine was the standard for both determinations. Unless the oligosaccharides with terminal glucosamine give a ratio of reducing groups to amino sugar much higher than that obtained from glucosamine itself, glucuronic bonds must also have been split. That the extract contains a glycosidase able to split the uronic bond is supported by the observed hydrolysis of polygalacturonic acid.

The ratio of the increase in micromoles of periodate consumed to reducing



group formed during the 1st hour of incubation remained at about 15:1. This high ratio (glucose is 5:1 and sucrose is 7:1 by this procedure) must mean the following: first, that a sulfesterase is also present in the extract and, second, that oligosaccharides are the predominant products at this stage. When the incubation times were prolonged until no further increase in reducing groups occurred (even upon the addition of fresh enzyme), the ratio of periodate consumption to reducing groups never fell below 9:1, nor did the amount of reducing groups formed ever exceed 50 per cent of the theoretical value for complete hydrolysis to monosaccharides.

Several hyaluronidases have been described (7), one of which (testicular) is also able to hydrolyze chondroitin sulfate, but none is able to degrade heparin. The ability of the bacterial extract to catalyze the hydrolysis of all three substrates is, therefore, unique. A more detailed comparison of the enzymatic hydrolysis of heparin, hyaluronic acid, and chondroitin sulfate will be the subject of another communication.

#### SUMMARY

The degradation of heparin by extracts of acetone powders of *Flaobacterium heparinum* has been studied. It has been found that the extract contains a sulfamidase, a sulfesterase, and at least one glycosidase which, together, catalyze an extensive cleavage of heparin.

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# ENZYMATIC REDUCTION OF THE C-20 CARBONYL GROUP OF TETRAHYDROCORTISONE AND 17-HYDROXYPREGNANOLONE\*

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It is known that reduction of the carbonyl group at C-20 to a secondary hydroxyl group occurs in the course of the metabolism of progesterone and the adrenocortical hormones in man. During the past 4 years, several groups of investigators have observed this reduction when certain adrenocortical hormones or their metabolites are perfused through liver or incubated with surviving liver slices or homogenates derived from liver. It has been established chiefly from the work of Recknagel and Glenn (2) that the enzyme system which effects this reduction is largely present in the microsomes.

We have studied the reduction of the carbonyl group at C-20 of pregnane- $3\alpha,17\alpha,21$ -triol-11,20-dione (tetrahydrocortisone) and of pregnane- $3\alpha,17\alpha$ -diol-20-one (17-hydroxypregnanolone) in fortified, fractionated homogenates of rat liver. In this paper we wish to describe the isolation of the metabolite pregnane- $3\alpha,17\alpha,20\beta,21$ -tetrol-11-one ( $\beta$ -cortolone) (3) after incubation of tetrahydrocortisone with such a homogenate and the isolation of the metabolites pregnane- $3\alpha,17\alpha,20\alpha$ -triol and its C-20 epimer after a similar incubation of 17-hydroxypregnanolone.

## Materials and Methods

*Preparation and Differential Centrifugation of Liver Homogenates*—Male albino rats weighing from 200 to 250 gm were decapitated, and the livers were excised rapidly and chilled in crushed ice. 5 gm of liver were ground for 30 seconds with 22.5 ml of the chilled Krebs phosphosaline buffer, pH 7.4 (4), in a chilled Potter homogenizer with a Teflon pestle.

Nuclei and cell debris were removed by centrifuging at  $600 \times g$  for 10 minutes in a refrigerated centrifuge. The supernatant solution ( $S_1$ ) was then centrifuged in a Servall centrifuge at  $6000 \times g$  for 30 minutes in a cold room to separate the mitochondria. When the mitochondria were required for an experiment, they were again homogenized in a little buffer, centrifuged at  $6000 \times g$  for 15 minutes, and suspended in sufficient buffer to make the final volume equal to that of  $S_1$ . The supernatant solution

\* A preliminary account has appeared (1)

remaining after the removal of the mitochondria was designated  $S_2$ . It contains the microsomes plus all of the soluble components and was the preparation used in most of the experiments. When required, the microsomes were separated in a Spinco centrifuge at  $100,000 \times g$  for 30 minutes and suspended in a volume of buffer sufficient to make the final volume equal to  $S_2$ . The pink microsome-free supernatant solution was designated  $S_3$ . It was difficult to remove all the microsomes from the mitochondrial fraction, but the preparation of microsomes appeared to contain few mitochondria.

*Conditions of Incubation and Determination of Tetrahydrocortisone*—In the small scale experiments, 200  $\gamma$  of free tetrahydrocortisone in 1 ml of the Krebs buffer were incubated in a Dubnoff apparatus at  $37^\circ$  with 1 ml of rat liver homogenate or fractionated homogenate in the presence of  $10^{-3}$  M reduced diphosphopyridine nucleotide (DPNH), 0.01 M sodium fumarate, and 0.04 M nicotinamide. The molar concentrations given are those of each substance in the complete incubation mixture, which had a final volume of 2.6 ml. After 2 hours incubation, 10 ml of ethanol were added to the flask, and 30 minutes later the contents were centrifuged. In each experiment a duplicate unincubated control set of flasks was prepared, to which ethanol was added immediately after the addition of the homogenate.

The tetrahydrocortisone in the supernatant solution was determined by the method of Porter and Silber (5). In each case 1 ml aliquots of the solution were treated with the incomplete (phenylhydrazine-lacking) reagent as well as with the complete reagent, and the corrected optical density thus obtained was used to calculate the degree of reduction of the carbonyl group at C-20.

In the large scale incubations, 24 gm of rat liver were homogenized in 90 ml of the Krebs buffer containing 500  $\gamma$  of free tetrahydrocortisone per ml. 50 ml of  $S_2$  obtained by the usual procedure were added to a flask containing 40 ml of the above tetrahydrocortisone solution, 15 ml of 0.13 M sodium fumarate solution containing 150 mg of nicotinamide, and 15 ml of the above tetrahydrocortisone solution containing 130 mg of diphosphopyridine nucleotide (DPN). The mixture was incubated for 3 hours at  $37^\circ$  with gentle shaking (gas phase, nitrogen) and was then diluted with 800 ml of acetone. After 30 minutes the suspension was filtered, the acetone was removed *in vacuo*, and the aqueous residue was extracted four times with *n*-hexane and six times with ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness *in vacuo*, and the residue was taken up in 2 ml of ethanol and 25 ml of methylene chloride. This solution was extracted eight times with water and then discarded. The combined aqueous phases were extracted six times with

ethyl acetate, which was then evaporated to dryness *in vacuo*. The residue was distributed in a 50 tube Craig all-glass countercurrent machine with the system ethyl acetate-isooctane-methanol-water (3:1:1:3). Provisional identification of the substances present was made by paper chromatography, and fractions which appeared to contain the same substance were combined and crystallized.

*Conditions of Incubation of 17-Hydroxypregnanolone*—Preliminary experiments were carried out with the system used for the incubation of tetrahydrocortisone, except that the quantities of all of the components were doubled. The steroid was added in a small volume of propylene glycol. After 2 hours incubation 10 ml of ethanol were added, and 30 minutes later the suspension was centrifuged. The clear fluid was decanted and, after the addition of 3 ml of water, the solution was extracted three times with *n*-hexane. The ethanol was removed with a stream of air, and the aqueous residue was diluted with water and extracted four times with ethyl acetate. Because no simple method for the quantitative analysis of the expected metabolites was available, the dried extracts were examined by paper chromatography.

In the large scale incubations, 40 mg of free 17-hydroxypregnanolone were dissolved in 0.7 ml of ethanol and 6 ml of propylene glycol in a small flask, and the solution was kept at 37° in the Dubnoff apparatus. Meanwhile, 24 gm of rat liver were homogenized with 90 ml of the Krebs buffer containing 270 mg of nicotinamide. 50 ml of S<sub>2</sub> prepared in the usual way were brought to 37° in a 1 liter Erlenmeyer flask. The propylene glycol solution of the steroid was then added slowly, and the liquids were mixed thoroughly. An additional 6 ml of propylene glycol were used to complete the transfer of the steroid. Finally a solution of 150 mg of DPN in 10 ml of buffer, warmed to 37°, was added to the flask. After incubation for 3 hours, the contents of the flask were diluted with 700 ml of acetone. 30 minutes later the suspension was filtered, and the filtrate was concentrated to a small volume *in vacuo*. After sufficient ethanol was added to the aqueous residue to adjust it to 70 per cent ethanol by volume, the solution was extracted four times with *n*-hexane. Most of the ethanol was removed with a stream of air, and the aqueous residue was extracted four times with ethyl acetate, which was then taken to dryness *in vacuo*. The residue at this stage was generally a green oil which was adsorbed on sufficient Celite to give a dry powder. The metabolites were then separated from each other and from the unchanged 17-hydroxypregnanolone by partition chromatography on a silica gel column with the system ethyl acetate-isooctane-methanol-water (200:400:180:420). 95 gm of activated silica gel<sup>1</sup> were shaken with 35 ml of the stationary

<sup>1</sup> Grade 922 silica gel, 200-270 mesh, from the Davison Chemical Company, Balti-

phase and, after it had been cooled, the treated gel was made into a thick suspension with the mobile phase and packed with a plunger into a 20 X 570 mm column. The Celite preparation of the extract was added to the top of the column, which was then developed with the mobile phase. The effluent was collected fractionally in volumes of 10 ml at a rate of 10 ml per hour.

*Paper Chromatography*—Cylindrical glass tanks 12 inches in diameter and 24 inches high were used, and the trough was supported on a metal or glass frame suitable for descending chromatography. Before use, the tank was lined with filter paper, and approximately 150 ml each of the stationary and mobile phases were poured in the tank, care being taken to wet the lining paper thoroughly. The remaining 50 ml of each phase were kept together in a small separatory funnel. Strips for chromatography were cut from Whatman No. 1 paper and, after the steroid solutions had been applied, the papers were equilibrated overnight in the tank before the mobile phase was added.

Aliquots of extracts from the tetrahydrocortisone incubations were chromatographed with the system ethyl acetate-isooctane-methanol-water (150:50:100:100), and the steroids were located by dipping the developed chromatograms in a 4 per cent solution of phosphomolybdic acid (PMA) in ethanol and heating them for 10 minutes at approximately 90°. In addition, tetrahydrocortisone was detected by dipping the developed chromatogram into a freshly prepared solution of equal volumes of 0.2 per cent aqueous triphenyltetrazolium chloride and 10 per cent NaOH in 60 per cent aqueous methanol (TPTZ). The metabolite was detected by a modification of the Bush (6) method for the detection of 17,20 diol. The developed chromatograms were sprayed with a 1 per cent solution of  $\text{HIO}_4 \cdot 2\text{H}_2\text{O}$  in 0.4 N  $\text{H}_2\text{SO}_4$  in 75 per cent aqueous ethanol. The paper was allowed to hang without drying for 30 minutes and was then dried in a current of air at room temperature and sprayed with a solution of 0.1 N NaOH in 80 per cent aqueous ethanol. After being dried again at room temperature, the 17-ketosteroid produced was detected by spraying the paper with a freshly prepared solution of equal volumes of 15 per cent KOH in absolute ethanol and 0.2 per cent metadinitrobenzene in absolute ethanol.

Aliquots of extracts obtained from the 17-hydroxypregnanolone incubations were chromatographed with the system toluene-isooctane-methanol-water (150:50:160:40). The 17-hydroxypregnanolone present was detected with fair success by dipping the developed chromatogram

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more, was washed repeatedly with distilled water by decantation. After the gel was dried in air, it was screened, and the 200-250 mesh fraction was washed and dried before and activated by being heated at 120° for 8 to 12 hours.

in a 15 per cent solution of phosphotungstic acid in ethanol and heating it for 20 minutes at 90°. The metabolites pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol and its C-20 epimer were detected with the trichloroacetic acid reagent (7)

### Results

*Incubation of Tetrahydrocortisone*—The unfortified whole rat liver homogenate showed little or no activity when incubated with tetrahydrocortisone but, when nicotinamide was added, about 25 per cent of the steroid was reduced at C-20 as judged by the Porter-Silber determination

TABLE I  
*Reduction of C-20 Carbonyl Group of Tetrahydrocortisone  
by Various Fractions of Rat Liver Homogenates*

The conditions and components other than the enzyme source are those given in the text for small scale tetrahydrocortisone incubations

Experiment No	Washed mito chondria	Microsome	Fraction S <sub>2</sub>	Fraction S <sub>3</sub>	Boiled fraction S <sub>2</sub>	Per cent reduction of tetrahydrocortisone
	<i>ml</i>	<i>ml</i>	<i>ml</i>	<i>ml</i>	<i>ml</i>	
1	1					5 3
	1			1		20 7
			1			48 7
2	1			1		15 7
				1		4 5
			1			65 7
3		1				22 5
		1			1	30 7
		1		1		62 3

The addition of DPNH to the preparation increased the reduction to about 50 per cent, and the addition of sodium fumarate resulted in a further small increase

Diphosphopyridine nucleotide, triphosphopyridine nucleotide (TPN), and its reduced form (TPNH) could be substituted for DPNH without appreciable change in the activity of the whole homogenate or the S<sub>2</sub> fraction but, since DPN is the least expensive of these nucleotides, it was used in most of the large scale incubations

Sodium fumarate could be replaced by the sodium salts of  $\alpha$ -ketoglutaric acid, isocitric acid, and oxalacetic acid without appreciable change in the activities of the whole homogenate or the S<sub>2</sub> fraction, but sodium fumarate was invariably used in these experiments

Experiment 1, Table I, shows the activities of the mitochondria and of

the mitochondria plus  $S_2$  compared with the activity of  $S_2$ , and demonstrates that the greater part of the activity is in the  $S_2$  (microsome plus supernatant solution) fraction. Experiment 2 demonstrates the relative inactivity of the  $S_3$  fraction, and Experiment 3 gives the activities of the microsome alone and in combination with  $S_3$  and with boiled  $S_3$ . The original or reconstituted  $S_2$  fractions have always been the most active preparations.

As a preliminary to the large scale experiments, 4 mg of tetrahydrocortisone were incubated with proportionately larger amounts of added factors and  $S_2$ . The crude extract was dissolved in 1 ml of methanol, 25  $\mu$ l volumes were chromatographed, and the results were assessed as indicated in Table II. The metabolite is the non-reducing substance

TABLE II

*Paper Chromatography of Extracts from Incubation of Tetrahydrocortisone*

The enzyme source was Fraction  $S_2$  from rat liver homogenate. For the conditions and other components, see the text.

Sample chromatographed	$R_F$ values and tests used		
	PMA	Periodate oxidation plus Zimmermann reaction	TPTZ
Extract of unincubated mixture Tetrahydrocortisone (control)	0.77, 0.88 0.76	None "	Not done " "
Extract of incubated mixture Tetrahydrocortisone (control)	0.49, 0.71, 0.90 0.72	0.50 None	0.70 0.75

which has an  $R_F$  value of 0.49 to 0.50 and which gives a 17-ketosteroid on oxidation with periodic acid. The system used does not resolve mixtures of cortol,  $\beta$ -cortol, cortolone, and  $\beta$ -cortolone (3), the  $R_F$  values for these steroids were in the range 0.48 to 0.53. Aliquots of the extracts were chromatographed also in systems suitable for the detection of 17-ketosteroids, but none were found.

In one of several large scale experiments, 65 mg of tetrahydrocortisone were incubated in the system and under the conditions described under "Materials and methods." Countercurrent distribution of the partially purified extract gave two well separated components. This was confirmed by paper chromatography of aliquots of the contents of a number of tubes. The contents of tubes 8 through 26 gave a crystalline residue which, when combined, weighed 18.9 mg. Two crystallizations from methanol gave fine needles which melted at 261–262°. On admixture with an authentic sample of  $\beta$ -cortolone (m.p. 260–261°) (3) the melting point was not

depressed The triacetate, prepared in the usual way with acetic anhydride and pyridine, melted at  $202-203^{\circ}$  On admixture with authentic  $\beta$ -cortolone triacetate (m p  $202-204^{\circ}$ ) (3), the melting point was unchanged In addition, the infrared spectra of the isolated and authentic triacetates were identical The second, more mobile component proved to be unchanged tetrahydrocortisone

*Incubation of 17-Hydroxypregnanolone*—The preliminary incubation experiments were evaluated as described under "Materials and methods" When the developed chromatograms which represented either the incubated or unincubated (control) vessels were treated with the phosphotungstic acid reagent, it was noted that the 17-hydroxypregnanolone had an  $R_F$  value of 0.61 in the described system The trichloroacetic acid reagent gave positive results only when applied to the developed chromatograms from the incubated vessels Two discrete spots which gave a blue fluorescence under ultraviolet light (with a source emitting at approximately  $366\text{ m}\mu$ ) were noted This indicated the presence of substances which had  $R_F$  values of 0.35 and 0.43, respectively

In the large scale experiment, 40 mg of 17-hydroxypregnanolone were incubated with the  $S_2$  fraction from rat liver homogenate under the conditions described under "Materials and methods" Partition chromatography of the partially purified extract on a silica gel column gave the following results 24 mg of unchanged 17-hydroxypregnanolone were eluted in Fractions 7 through 10 The next component, which was eluted in Fractions 14 through 19, gave 8.4 mg of cubic crystals, m p  $220-221^{\circ}$  On admixture with an authentic sample of pregnane- $3\alpha,17\alpha,20\beta$ -triol (m p  $217-217.5^{\circ}$ ), the melting point was  $218-219^{\circ}$  Acetylation of the isolated metabolite, followed by crystallization from aqueous methanol, gave needles which melted at  $198-200^{\circ}$  When mixed with an authentic sample of pregnane- $3\alpha,17\alpha,20\beta$ -triol-3,20-diacetate (m p  $198.5-199^{\circ}$ ), the melting point was not depressed In addition, on paper chromatography in the described system, the  $R_F$  value of the isolated free steroid corresponded to that of authentic pregnane- $3\alpha,17\alpha,20\beta$ -triol and gave a similar blue fluorescence with the trichloroacetic acid reagent The second metabolite was eluted in Fractions 20 through 23 Crystallization from methanol gave 3.2 mg of needles which melted at  $254.5-255.5^{\circ}$  The acetate melted at  $159-161^{\circ}$ , and, when it was mixed with authentic pregnane- $3\alpha,17\alpha,20\alpha$ -triol-3,20-diacetate (m p  $158-159^{\circ}$ ), the melting point was not depressed The isolated free steroid had the same  $R_F$  value as authentic pregnane- $3\alpha,17\alpha,20\alpha$ -triol in the system described and gave a similar blue fluorescence when the paper was treated with the trichloroacetic acid reagent

Two similar experiments were carried out in which rabbit liver served



as the enzyme source. In the first of these TPN was used, and 14.0 mg of pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol and 9.4 mg of its C-20 epimer were obtained. In the second, DPN was added, and 5.3 mg of pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol and 11.4 mg of its C-20 epimer were recovered.

#### DISCUSSION

The enzymatic conversion of tetrahydrocortisone to  $\beta$ -cortolone reported in this paper is in accord with the demonstration that significant amounts of  $\beta$ -cortolone were excreted after the administration of tetrahydrocortisone to a man (8). Earlier, Fukushima and his associates (3) reported the isolation of  $\beta$ -cortolone and three related steroids from the urine of a patient given adrenocorticotropin. These steroids were regarded as metabolites of hydrocortisone, and it now appears from our results that, in the metabolic sequence, tetrahydrocortisone is an immediate precursor of  $\beta$ -cortolone.

The recovery of pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol and its C-20 epimer after the incubation of 17-hydroxypregnanolone with rat liver homogenate is in agreement with the general view that, in man, pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol is a metabolite of 17-hydroxypregnanolone, which in turn is derived from 17-hydroxyprogesterone. Previously, 17-hydroxypregnanolone has been recovered from the urine of normal humans and from the urine of patients with adrenocortical lesions (see Appleby and Nislymberski (9) for review) and recently we have shown that the oral administration of 17-hydroxypregnanolone and 17-hydroxyprogesterone to a man is followed by the excretion of pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol and a small amount of its C-20 epimer.

These results were compared with those of Recknagel and Glenn (2) and Hubener and his associates (10) with respect to the coenzyme requirement. The former investigators incubated cortisone with rat liver microsomal preparations and showed that reduction at C-20 occurs only in the presence of TPNH or a TPNH-generating system. The present results are in essential agreement with these in that they show that a phosphopyridine nucleotide coenzyme is required. The observation that DPN, TPN, and their dihydro forms were equally active may be interpreted to mean either that the reaction is not wholly specific for TPNH or that conversion of the diphosphopyridine nucleotides to the triphosphopyridine nucleotides occurred in the presence of the  $S_3$  fraction. Hubener and his associates (10) incubated a number of steroids with whole or fractionated rat liver homogenates and decided that TPN is not a cofactor. A number of explanations may be advanced to account for this conclusion. It seems likely that, since that ratio of steroid to tissue was high and the homogenate was relatively dilute, sufficient TPNH was present to permit a limited

reaction In their fractionation experiments, they found that the  $S_2$  fraction was less active than the whole homogenate and that the microsomes alone were inactive The loss of activity in these preparations may have been due either to destruction of the endogenous TPNH-generating system or its removal during the fractionation procedure Further, the apparent ineffectiveness of the addition of TPNH to the homogenate could have been due to the omission of nicotinamide which is customarily added to *in vitro* systems to inhibit nucleotidase activity

These considerations do not account for the fact that Hubener and his associates obtained cortolone after the incubation of tetrahydrocortisone, whereas only  $\beta$ -cortolone was isolated in these experiments A survey of the literature revealed other similarly conflicting results Table III summarizes the results of a number of investigators in terms of the steroids incubated or perfused, the enzyme source employed, and the metabolites formally identified It lists only those metabolites in which reduction has occurred at C-20 and in which the configuration at that position has been determined Since several techniques were used to fractionate the tissue and various methods were employed to isolate the metabolites, it is difficult to compare the results in detail If the comparison is based on the structures of the metabolites isolated, it is clear that, although both epimers have been obtained in some instances, the  $20\beta$  isomer has been isolated in the majority of cases, regardless of which steroid or enzyme source was employed Hubener and his associates (10) believe that a factor of substrate specificity is involved Relative to the reduction of the C-20 carbonyl group, they have concluded that the  $20\beta$  epimer is formed predominantly when the substrate has an  $11\beta$  hydroxyl group, and that the  $20\alpha$  epimer is the chief reduction product when a carbonyl group or no functional group is present at C-11 Inspection of Table III shows that, although some of the results (11, 13, 16, 17) support this conclusion, others (12, 14, 15) do not We believe that it is possible to account for some of the apparently conflicting results on other as yet hypothetical grounds In view of the evidence that the known pyridine nucleotide dehydrogenases show complete stereospecificity for the asymmetric carbon atom of the substrate (18), it seems certain that liver contains both a  $20\alpha$ - and a  $20\beta$ -hydroxysteroid dehydrogenase This suggestion is supported (a) by the demonstration of Talalay and Marcus (19) that extracts of *Pseudomonas saccharophila* contain both a  $3\alpha$ - and a  $3\beta$ -hydroxysteroid dehydrogenase, (b) by the results of Forchielli (12), who isolated both  $\Delta^4$ -pregnene- $17\alpha$ , $-20\alpha$ , $21$ -triol-3-one and its C-20 epimer after incubation of  $\Delta^4$ -pregnene- $17\alpha$ , $21$ -diol-3, $20$ -dione with rat liver homogenate, and (c) by our recovery of both of the pregnanetriols epimeric at C-20 after incubation of  $17\alpha$ -hydroxypregnanolone with rat liver homogenate If it can be postulated

that the properties and intracellular localization of the 20 $\alpha$ - and the 20 $\beta$  hydroxysteroid dehydrogenases differ slightly, it seems probable that their relative activities in the several types of preparations would be different

TABLE III  
*Enzymatic Reduction of C-20 Carbonyl Group of Various Steroids*

Steroid studied	Enzyme source	Metabolite identified	Bibliographic reference
Deoxycorticosterone	Hog liver homogenate	$\Delta^4$ -Pregnene-20 $\alpha$ ,21-diol-3 one	(11)
$\Delta^4$ -Pregnene-17 $\alpha$ ,21-diol-3,20-dione	Rat liver homogenate	Allopregnane-3 $\beta$ ,17 $\alpha$ ,20 $\beta$ ,21-tetrol $\Delta^4$ -Pregnene-17 $\alpha$ ,20 $\beta$ ,21-triol-3-one $\Delta^4$ -Pregnene-17 $\alpha$ ,20 $\alpha$ ,21-triol-3-one	(12)
$\Delta^4$ -Pregnene-17 $\alpha$ ,21-diol-3,20-dione	Rat liver homogenate	$\Delta^4$ -Pregnene-17 $\alpha$ ,20 $\alpha$ ,21-triol 3-one	(13)
$\Delta^4$ -Pregnene-17 $\alpha$ ,21-diol-3,20-dione	Hog liver homogenate	$\Delta^4$ -Pregnene-17 $\alpha$ ,20 $\alpha$ ,21-triol 3-one	(11)
$\Delta^4$ -Pregnene-17 $\alpha$ ,21-diol-3,20-dione	Rat liver homogenate	$\Delta^4$ -Pregnene-17 $\alpha$ ,20 $\alpha$ ,21-triol 3-one	(10)
Cortisone	Rat liver perfusion	Allopregnane-3 $\beta$ ,17 $\alpha$ ,20 $\beta$ ,21-tetrol-11-one $\Delta^4$ -Pregnene-17 $\alpha$ ,20 $\beta$ ,21-triol 3,11-dione $\Delta^4$ -Pregnene-11 $\beta$ ,17 $\alpha$ ,20 $\beta$ ,21-tetrol-3-one	(14, 15)
Tetrahydrocortisone	Rat liver homogenate	Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ ,21-tetrol-11-one	(10)
"	Rat liver homogenate	Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ ,21-tetrol-11-one	This paper (10)
Hydrocortisone	Rat liver homogenate	$\Delta^4$ -Pregnene-11 $\beta$ ,17 $\alpha$ ,20 $\beta$ ,21-tetrol-3-one $\Delta^4$ -Pregnene-17 $\alpha$ ,20 $\beta$ ,21-triol 3,11-dione	
Hydrocortisone-4-C <sup>14</sup>	Rat liver perfusion	Allopregnane-3 $\beta$ ,11 $\beta$ ,17 $\alpha$ ,20 $\beta$ ,21-pentol $\Delta^4$ -Pregnene-11 $\beta$ ,17 $\alpha$ ,20 $\beta$ ,21-tetrol-3-one	(16)
Pregnane-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,21-tetrol-20-one	Rat liver homogenate	Pregnane-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,20 $\beta$ ,21-pentol	(10)
Pregnane-3 $\alpha$ ,17 $\alpha$ -diol-20-one	Rat liver homogenate	Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol Pregnane 3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ triol	This paper (17)
Progesterone	Rabbit liver homogenate	Pregnane 3 $\alpha$ ,20 $\alpha$ diol	

As an alternative suggestion, Dr Kurt Isselbacher has pointed out to us that some of the preparations may have contained an isomerase capable of effecting inversion of the hydroxyl group at C-20

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#### SUMMARY

1 Tetrahydrocortisone and 17-hydroxypregnanolone have been incubated with a fractionated homogenate of rat liver in the presence of a pyridine nucleotide coenzyme, nicotinamide, and sodium fumarate.

2  $\beta$ -Cortolone was obtained from tetrahydrocortisone and pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol, and its C-20 epimer from 17-hydroxypregnanolone.

3 These results have been compared with those obtained by other investigators in this field.

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# STEROIDS OF GUINEA PIG URINE\*

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WITH THE TECHNICAL ASSISTANCE OF ARLINE M TILLOTSON

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After Nadel and Schneider (1) had indicated the presence of cortisol in the urine of normal guinea pigs, some aspects of the nature of the adrenocortical secretion in the guinea pig in addition to the effects of adrenocorticotrophic hormone (ACTH) thereon were elucidated by Burstein *et al* (2, 3). In addition, the effect of an ascorbic acid-deficient diet on the excretion of urinary corticosteroids in the guinea pig has also been studied in the same laboratories (3). Various workers have reported the presence of 17-ketosteroids in the urine of normal, ascorbic acid-deficient, and ACTH-stimulated guinea pigs as detected by the Zimmermann reaction, but no identification of these materials has been reported (4-6). This communication is concerned with the identification of  $3\alpha,11\beta$ -dihydroxyetiocholan-17-one,  $3\alpha,11\beta$ -dihydroxyandrostan-17-one,  $3\alpha$ -hydroxyandrostane-11,17-dione,  $3\alpha$ -hydroxy- $\Delta^9$  etiocholen-17-one and  $3\alpha$ -hydroxyetiocholan-17-one as a mixture, and  $3\alpha$ -hydroxy- $\Delta^9$  androsten-17-one as the main 17-ketosteroid components of urine from ACTH-stimulated guinea pigs. In addition, the structure of the previously described compound steroid IIa (2, 7) has been elucidated as being  $2\alpha,11\beta,17\alpha,21$ -tetrahydroxy- $\Delta^4$ -pregnene-3,20-dione ( $2\alpha$ -hydroxycortisol). Burstein (8) has independently established the structure of this compound.

## Methods

Nineteen normal adult male guinea pigs (600 to 700 gm), obtained from the Rockland Farms, New City, New York, were placed in metabolism cages, and the urine was collected for 7 days as previously described (1). The animals were permitted access to food and water for a period of 3 hours each day, and the urine was collected during the subsequent 21 hours. After the daily feeding and watering period, and prior to being placed in the metabolism cages, each guinea pig was injected intraperitoneally with 8 iu of ACTH (ACTHAR Gel Armour), the administration of which was repeated once each day for 7 days.

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The 7 day pooled urine was extracted with ethyl acetate as previously described (1), and yielded a neutral extract A. Subsequently, neutral extract B was obtained after an 8 minute hot acid hydrolysis (15 per cent concentrated HCl by volume) of the urine and reextraction with ethyl acetate.

In order to minimize the effects of excessive pigments and extraneous non-steroidal material on the subsequent paper chromatograms, extracts A and B were chromatographed on silica gel columns as recommended by Romanoff (9). Nine fractions were collected, the solvents used to elute the column being, for Fraction 1, benzene, Fractions 2 to 4, benzene-ethyl ether mixtures in ratios 2:1, 1:1, and 1:2, respectively, Fraction 5, ether, Fraction 6, ethyl acetate, Fraction 7, ethyl acetate-acetone 1:1, Fraction 8, acetone, and Fraction 9, methanol. The volumes of Fractions 1 to 9 and the amount of silica gel used for preparation of the columns were dependent on the weight of crude extracts A and B (cf. Romanoff (9)). The corticosteroids, as ascertained by control runs, and the subsequent paper chromatography of each eluted fraction (1 to 9) were shown to be eluted in Fractions 6 to 8, while 17-ketosteroids were eluted in Fractions 3 to 5. No corticosteroids were demonstrated in any of the fractions obtained from neutral extract B, although trace amounts of 17-ketosteroids were detected in Fractions 3 to 5 of neutral extract A. The latter fractions were combined with Fractions 3 to 5 of neutral extract B. The purified neutral extracts thus obtained were designated as Fractions A<sub>1</sub> and B<sub>1</sub>.

Fractions A<sub>1</sub> and B<sub>1</sub> were chromatographed on paper with conventional solvent systems (10-12). The extracts were chromatographed first in the chloroform-formamide system, and the 17 hour runoff was treated in the same system for 7 hours. The 7 hour runoff was chromatographed in the toluene-propylene glycol system and the 40 hour runoff in the ligroin-propylene glycol system for 120 hours. The 120 hour ligroin-propylene glycol runoff was chromatographed in the same system for 40 hours and the runoff partitioned again in the ligroin-propylene glycol system for 7 hours. The 7 hour ligroin-propylene glycol runoff was not collected. Individual corticosteroid zones were detected by use of the blue tetrazolium reagent (BF) and by examination of the chromatograms under ultraviolet light (Mineralight, Ultra-Violet Products, Inc., South Pasadena, California). The 17 ketosteroid zones were detected by development of the chromatograms with the Zimmermann reagent (10).

#### EXPERIMENTAL

##### *Steroids of Fraction A<sub>1</sub>*

The 17 hour chloroform-formamide system resolved neutral Fraction A<sub>1</sub> into six zones which were detected by examination of the chromatograms

under ultraviolet light. Four of these zones also reduced BT. No additional zones which absorbed ultraviolet light or reacted with the BT or Zimmermann reagent were detected on the chromatograms run in the other systems. The positions occupied by these zones on the chromatograms were almost identical to those already reported by Burstein *et al* (3). Eluates of our Zones I, IV, and VI were identified by methods previously reported (3) and shown to be  $6\beta, 11\beta, 17\alpha, 21$ -tetrahydroxy- $\Delta^4$ -pregnene-3,20-dione ( $6\beta$ -hydroxycortisol),  $11\beta, 17\alpha, 20\alpha, 21$ -tetrahydroxy- $\Delta^4$ -pregnen-3-one, and cortisol, respectively. Our Zone II, which had the same characteristics as steroid IIa of Burstein and Dorfman (7), was identified as  $2\alpha, 11\beta, 17\alpha, 21$ -tetrahydroxy- $\Delta^4$ -pregnene-3,20-dione ( $2\alpha$ -hydroxycortisol).

*2 $\alpha$ -Hydroxycortisol (Zone II)*—The eluates of Zone II, obtained from seven chromatograms run for 17 hours in the chloroform-formamide system, were combined and brought to dryness *in vacuo*. The only residue was dissolved in a mixture of ethanol-ethyl acetate (1:1) and crystallized from this solvent, thus affording a few mg of white rhombic plates melting at 190–191° (uncorrected, Kofler micro hot stage). The substance reduced BT, and had a sulfuric acid chromogen spectrum identical with steroid IIa-ac of Burstein and Dorfman (7) and an ultraviolet absorption maximum in methanol at  $242\text{ m}\mu$ . Since the acetylated product of Zone II (m.p. 227°,  $E_{1\text{ cm}}^{1\%}$  317,  $\epsilon_{\text{max}}$  = 14,600) exhibited a mobility behavior reminiscent of  $\alpha$ -hydroxylated steroids after acetylation, an authentic sample of  $2\alpha, 17\alpha, 21$ -trihydroxy- $\Delta^4$ -pregnene-3,11,20-trione<sup>1</sup> ( $2\alpha$ -hydroxycortisone) was obtained and acetylated. After the acetate of the eluate from Zone II had been oxidized with chromic acid for 1 minute at room temperature (13), 250  $\gamma$  of the reaction product and 200  $\gamma$  of  $2\alpha$ -hydroxycortisone diacetate were chromatographed singly and admixed in the toluene-propylene glycol system for 7 hours. The positions occupied by  $2\alpha$ -hydroxycortisone diacetate and oxidized acetate from Zone II on the chromatograms, as ascertained by examination of the chromatograms with ultraviolet light, and their reaction with the BT reagent were identical. No separation of the two compounds was observed in the mixed chromatogram. Elution of all three chromatograms and careful infrared spectroscopic analysis of the eluates yielded infrared spectra which were identical for all three eluates: hydroxyl at 3600, ester carbonyl at 1750, C-20 carbonyl at 1712, conjugated carbonyl at 1695, a double bond at 1620, and acetate at 1240 and 1222  $\text{cm}^{-1}$ . Sulfuric acid chromogen spectra of 100  $\gamma$  of authentic  $2\alpha$ -hydroxycortisone diacetate and oxidized acetate from Zone II were identical and possessed absorption maxima at 317, 405 (I), and 462  $\text{m}\mu$  (I), and minima at 240, 385 (I), and 455  $\text{m}\mu$  (I).

<sup>1</sup> Kindly donated by Dr A. Zaffaroni of Syntex, S. A., Mexico, D. F.



*Steroids of Fraction B<sub>1</sub>*

Chromatographic resolution of the steroid components of Fraction B<sub>1</sub> was accomplished in the ligroin-propylene glycol system. In all, four zones (I to IV) which reacted with the Zimmermann reagent were rendered visible on chromatograms run in the ligroin-propylene glycol system for 120 hours (L-P-G-120), and two zones (I to II) on the subsequent chromatograms run in the same system for 40 hours (L-P-G-40). In Fig 1 is a diagrammatic presentation of the separation observed after development of the chromatograms with the Zimmermann reagent.

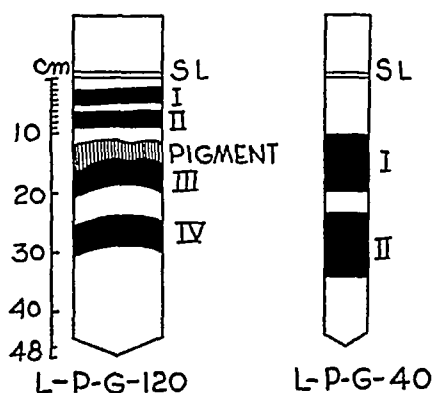


FIG 1 Zones on chromatograms run in ligroin-propylene glycol for 120 hours (L-P-G-120) and ligroin-propylene glycol for 40 hours (L-P-G-40) and detected with the Zimmermann reagent. Zone L-P-G-120-I was identified as  $3\alpha,11\beta$ -dihydroxyetiocholan-17-one, Zone L-P-G-120-II as  $3\alpha,11\beta$ -dihydroxyandrostan-17-one, Zone L-P-G-120-III was unknown, Zone L-P-G-120-IV as  $3\alpha$ -hydroxyandrostane-11,17-dione, Zone L-P-G-40-I as a mixture of  $3\alpha$ -hydroxy- $\Delta^9$ -etiocholan-17-one and  $3\alpha$ -hydroxyetiocholan-17-one, Zone L-P-G-40-II as  $3\alpha$ -hydroxy- $\Delta^9$ -androsten-17-one. The location of the zones is indicated on the scale in cm from the starting line (S L).

*$3\alpha,11\beta$ -Dihydroxyetiocholan-17-one (L-P-G-120-I)*—Elution of Zone L-P-G-120-I, followed by acetylation, yielded a product which, when chromatographed in the ligroin-propylene glycol system for 40 hours singly or admixed with  $3\alpha,11\beta$ -dihydroxyetiocholan-17-one-3-acetate, showed identical chromatographic behaviors. Elution of L-P-G-120-I acetate and subsequent infrared analysis confirmed the identity of this compound.

*$3\alpha,11\beta$ -Dihydroxyandrostan-17-one (L-P-G-120-II)*—The material in Zone L-P-G-120-II was acetylated and chromatographed in the ligroin-propylene glycol system for 24 hours. Mixed chromatograms of the eluate of L-P-G-120-II acetate with pure  $3\alpha,11\beta$ -dihydroxyandrostan-17-one-3-acetate showed no separation. The infrared spectrum of the standard  $3\alpha,11\beta$ -dihydroxyandrostan-17-one-3-acetate was identical with that of L-P-G-120-II acetate.

*3 $\alpha$ -Hydroxyandrostane-11,17-dione (L-P-G-120-IV)*—The identity of Zone L-P-G-120-IV was established in the same manner as for Zones L-P-G-120-I and II, that is, by comparison of the paper chromatographic mobility of its acetate, singly and admixed with standard 3 $\alpha$ -hydroxyandrostane-11,17-dione-3-acetate, and by infrared spectroscopy

*Mixture of 3 $\alpha$ -Hydroxy- $\Delta^9, 11$ -etiocholen-17-one and 3 $\alpha$ -Hydroxyetiocholan-17-one (L-P-G-40-I)*—L-P-G-40-I had the same mobility as 3 $\alpha$ -hydroxyetiocholan-17-one (etiocholanolone) and the corresponding unsaturated  $\Delta^9, 11$  analogue, and separation of these two substances could not be effected in mixed chromatograms. Since infrared spectroscopy of eluates of L-P-G-40-I showed certain similarities but over-all different infrared spectra from etiocholanolone and 3 $\alpha$ -hydroxy- $\Delta^9, 11$ -etiocholen-17-one, the possibility existed that L-P-G-40-I was a mixture of both of these compounds. That this was in fact the case was demonstrated by the analysis of the absorption spectrum of 100  $\gamma$  of Zone L-P-G-40-I in sulfuric acid (14) by a method developed by Savard.<sup>2</sup> The amount of etiocholanolone and 3 $\alpha$ -hydroxy- $\Delta^9, 11$ -etiocholen-17-one in L-P-G-40-I was found to be approximately 60 and 40 per cent, respectively, of the total Zimmermann-reacting material.

*3 $\alpha$ -Hydroxy- $\Delta^9, 11$ -androsten-17-one (L-P-G-40-II)*—The infrared spectra of eluates of Zone L-P-G-40-II and authentic 3 $\alpha$ -hydroxy- $\Delta^9, 11$ -androsten-17-one were identical.

#### DISCUSSION

The isolation of a hydroxylated compound at the C-2 position of the steroid nucleus has already been reported by Axelrod and Miller (15) in the form of 2 $\beta$ ,17 $\beta$ -dihydroxy- $\Delta^4$ -androsten-3-one after perfusion of the dog liver with testosterone. The isolation of 2 $\alpha$ -hydroxycortisol by Burstein (8) from the urine of guinea pigs, confirmed in our experiment, represents the second instance in which a steroid hydroxylated at the C-2 position is observed and indicates another new avenue to which steroids are directed, probably before they are metabolized further in the animal organism. The likelihood that the 2 $\beta$ -hydroxycortisol isomer also is excreted in the urine of the guinea pig is tenable in view of the fact that Zone III and its acetate found on the 17 hour chloroform-formamide chromatogram have mobility characteristics which are in accord with such a possibility.

Although the majority of the corticosteroids which are excreted in human urine are isolated as saturated ring A metabolites, in contradistinction, all corticosteroids which have been isolated at the present time from guinea pig urine possess the  $\Delta^4$ -3-keto conjugated double bond in ring A. The

<sup>2</sup> Savard, K, to be published. This method consists of the comparison of the sulfuric acid spectrum of the unknown with those obtained with mixtures of known composition of etiocholanolone and 3 $\alpha$ -hydroxy- $\Delta^9, 11$ -etiocholen-17-one.

fact that the tetrahydro compounds urocortisone and urocortisol (16) were not found in guinea pig urine after injection of ACTH or cortisol administration (7) suggests that corticosteroids are metabolized in a manner different from that in man. The isolation of urinary steroids hydroxylated at the C-2 and C-6 position of the steroid nucleus in the normal, ACTH- and cortisol-treated guinea pig (3, 7) may be consistent with the findings of Axelrod and Miller in the dog (15) that hydroxylation of testosterone precedes its breakdown or metabolism, and that oxidation is also the major initial alteration of corticosteroids in the guinea pig before the subsequent metabolic steps.

The 17-ketosteroid picture, however, is in accord with what has been found in man, namely, that the C-19 urinary steroids are of the ring A saturated variety. The presence of  $3\alpha$ -hydroxy- $\Delta^9$ , $^{11}$ -etiocholan-17-one and the corresponding unsaturated androstane compound is considered to be an artifact of acid hydrolysis by dehydration of  $3\alpha$ , $11\beta$ -dihydroxyetiocholan-17-one and  $3\alpha$ , $11\beta$ -dihydroxyandrostane-17-one, respectively.

The 11-oxygenated C-19-saturated steroids isolated from guinea pig urine are undoubtedly of adrenal origin, since testicular tissue *in vivo* contributes primarily C-19 11-deoxy type steroids to the pool of urinary steroids. However, it is not known at the present moment whether these C-19 11-oxygenated steroids are released as C<sub>19</sub>O<sub>3</sub> steroids by the adrenal or are the end products of corticosteroid metabolism, which are finally excreted in the urine.

#### SUMMARY

$2\alpha$ , $11\beta$ , $17\alpha$ , $21$ -Tetrahydroxy- $\Delta^4$ -pregnene-3,20-dione ( $2\alpha$ -hydroxycortisol),  $3\alpha$ , $11\beta$ -dihydroxyetiocholan-17-one,  $3\alpha$ , $11\beta$ -dihydroxyandrostane-17-one,  $3\alpha$ -hydroxyandrostane-11,17-dione,  $3\alpha$ -hydroxy- $\Delta^9$ , $^{11}$ -etiocholan-17-one and  $3\alpha$ -hydroxyetiocholan-17-one as a mixture, and  $3\alpha$ -hydroxy- $\Delta^9$ , $^{11}$ -androstane-17-one have been isolated from the urine of the guinea pig injected with adrenocorticotrophic hormone.

*Addendum*—Since the submission of this paper for publication, identification of Zone L-P-G-120-II has been accomplished and is  $3\alpha$ -hydroxyetiocholan-11,17-dione. Its identity was ascertained by infrared spectroscopy, mobility of the free and acetylated product on paper chromatograms with and without standard substance. The free compound, when crystallized from methanol, melted at 183-185°.

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# PURIFICATION AND SOME PROPERTIES OF PHOSPHORYLGLYCERIC ACID MUTASE FROM RABBIT SKELETAL MUSCLE\*

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The reversible conversion of 3-phosphoryl-D-glyceric acid (3PGA) into 2-phosphoryl-D-glyceric acid (2PGA) was first shown to occur in rabbit muscle extracts by Meyerhof and Kiessling (1) as one step in the pathway of glycolysis. These authors determined an equilibrium constant for this reaction in crude muscle extracts and showed that only the D(−) isomers of 2PGA and 3PGA were substrates. Although early attempts to purify the enzyme were unsuccessful (1, 2), Sutherland *et al* (3) obtained a partially purified preparation and demonstrated the participation of 2,3-diphosphorylglyceric acid in the reaction.

The present investigations have shown that the enzyme from rabbit muscle is quite stable to most purification procedures and a 90-fold purification has been obtained. This preparation was free from enolase, and a direct determination of the equilibrium of the mutase-catalyzed reaction was possible. No metal ion requirement could be demonstrated for this preparation, but the enzyme was inhibited by fluoride and heavy metal ions and by sulfhydryl reagents. As a step toward further study of the mechanism of action of this enzyme, the effects of compounds structurally related to phosphorylglyceric acid have been tested, both as substrates and as inhibitors.

## EXPERIMENTAL

### *Materials and Methods*

3-Phosphoryl-D-glyceric acid was obtained from the Schwarz Laboratories, Inc., as the barium salt. The elementary composition of this salt agreed well with the formula,  $\text{BaC}_3\text{H}_5\text{O}_7\text{P} \cdot 2\text{H}_2\text{O}$ , and the compound has the same mobility upon paper chromatography (4) as 3PGA isolated from yeast fermentation mixtures or 3PGA synthesized by the method of Ballou and Fischer (5). The material was free of inorganic phosphate and 2PGA.

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but the optical rotation,  $[\alpha]_D^{20}$ , was only  $-620^\circ$  as compared with a value of  $-745^\circ$  for authentic, natural, or synthetic D-3PGA as measured in 8 per cent ammonium molybdate. No explanation for this low value could be found. For the enzymatic studies, solutions of 3PGA were prepared by treatment of the barium salt with Dowex 50 ( $\text{Na}^+$ ), and the concentrations of 3PGA in these solutions were determined polarimetrically. This material did not require the addition of 2,3-diphosphorylglyceric acid for enzyme activity. 2-Phosphoryl-D-glyceric acid was synthesized by the method of Ballou and Fischer (6), and 2,3-diphosphoryl-D-glyceric acid was isolated by the procedure of Greenwald (7) and added routinely in all enzyme assays when synthetic substrates were present. Crystalline enolase was obtained by the method of Warburg and Christian (8). Protein was determined by the method of Lowry *et al.* (9).

Two types of assay of enzymatic activity were made.

**Assay A**—The mutase was coupled with crystalline enolase (3) under such conditions that the mutase reaction was the rate-limiting step in the sequence  $3\text{PGA} \rightarrow 2\text{PGA} \rightarrow \text{PEP}$ , and the formation of phosphoenolpyruvate (PEP) was followed by the increase in light absorption at  $240\text{ m}\mu$ . The 3.0 ml of assay mixture in a cuvette of 1 cm diameter contained  $5 \times 10^{-3}\text{ M}$   $\text{MgCl}_2$ ,  $3.3 \times 10^{-2}\text{ M}$  imidazole buffer at pH 7.0, excess crystalline enolase, and either  $2.0 \times 10^{-3}\text{ M}$  3PGA or mutase at the required dilution. The reaction was started by addition of the missing component (substrate or mutase) and the change in optical density at  $240\text{ m}\mu$  was followed in a Beckman model DUR recording spectrophotometer. The mutase preparation was diluted so that a change of 0.075 in optical density was obtained in approximately the 1st minute of the reaction. In this region, the rate of change of optical density was directly proportional to the amount of mutase present. Under the above conditions, 1 unit of enzyme activity is defined as that amount of enzyme which can form 0.1 mmole of PEP per minute (the molar extinction coefficient of PEP is assumed to be 1500).<sup>1</sup>

**Assay B**—Assays by the polarimetric method of Meyerhof and Schultz (10) also were made, particularly in activation and inhibition studies. Mixtures of  $2.5 \times 10^{-3}\text{ M}$  imidazole buffer and either  $1.8 \times 10^{-3}\text{ M}$  3PGA or  $0.9 \times 10^{-3}\text{ M}$  2PGA as substrate plus any agents that were to be tested as activators or inhibitors were equilibrated at  $37^\circ$ . The reaction was started by the addition of mutase, and 2.0 ml samples were removed at desired intervals. Each sample was added to 1.7 ml of 25 per cent (w/v) ammonium molybdate in a 5 ml volumetric flask. This served both to stop the reaction and to provide ammonium molybdate for the polarimetric measurement of 3PGA. The flasks were diluted to volume with

<sup>1</sup> C. E. Ballou, personal communication.

water and the optical rotation was measured with a Keston polarimeter attachment for the Beckman model D spectrophotometer or with a conventional polarimeter. With either polarimeter, the limit of accuracy was  $\pm 0.02^\circ$ . This variation led to a limit of error of 5 per cent in the studies in which the conversion from 2PGA to 3PGA occurred, and of 20 per cent when the reaction proceeded in the reverse direction.

### *Enzyme Purification*

*Extraction Step I*—The rabbit was anesthetized with Nembutal and bled from the neck. The back and hind leg muscles were removed quickly and placed on ice prior to passage through a chilled meat grinder. All subsequent steps were carried out at  $4^\circ$  unless otherwise stated. The ground tissue was extracted with 1.5 times its weight of cold distilled water for 15 minutes and then strained through gauze. The extraction was repeated for 10 minutes with an amount of water equal in weight to the ground tissue and the extract was again strained and then pressed in order to increase the amount of extract obtained.

*First Heat Denaturation Step II*—The combined extracts were collected in a 6 liter flask, placed in a large water bath at  $65^\circ$ , and swirled continuously so that the temperature of the extract rose to  $55^\circ$  in 8 to 10 minutes. A heavy precipitate formed at this temperature and the flask was kept in the bath for an additional 5 minutes while the temperature was allowed to reach but not to exceed  $60^\circ$ . The flask was transferred to an ice bath, cooled to below  $10^\circ$ , and the extract was centrifuged in a refrigerated centrifuge at 3000 r p m for 15 minutes. Any particles that floated in the supernatant fluid were removed by filtration through glass wool.

*Ammonium Sulfate Precipitation Step III*—Solid ammonium sulfate was slowly added with mechanical stirring to the cleared solution to give a final concentration of 550 gm per liter. After standing overnight, the mixture was centrifuged at 3000 r p m for 60 minutes. The turbid supernatant fluid was discarded and the precipitate was suspended in 50 ml of cold water. This suspension was dialyzed<sup>2</sup> against a 4 liter reservoir of water which was changed five times during an 18 hour period. The precipitate which remained was removed by centrifugation.

*Second Heat Denaturation Step IV*—Imidazole buffer, pH 7, and 3PGA were added to the clear supernatant fluid from Step III to give final concentrations of  $2 \times 10^{-2}$  M and  $2.5 \times 10^{-4}$  M, respectively. The addition of 3PGA was essential for stabilization of the mutase in subsequent purification steps. The procedure used for Step II was repeated, although the time required was much shorter and turbidity appeared at  $50^\circ$ . Care must be taken that the temperature does not exceed  $60^\circ$  or that the 5

<sup>2</sup> Dialysis bags were soaked in  $10^{-2}$  M Versene prior to use.



minute period of exposure above  $55^{\circ}$  is not extended. A heavy precipitate was removed by centrifugation at 10,000 r p m for 10 minutes.

*First Alcohol Fractionation Step V*—The supernatant fluid from Step IV was placed in an ice bath and 0.1 of its volume of polyethylene glycol (average molecular weight 400) was added. Then 95 per cent ethanol which had been cooled to  $-10^{\circ}$  was added from a burette with mechanical stirring and at such a rate that the temperature of the mixture never exceeded  $3^{\circ}$ . A total of 35 ml of ethanol was added per 100 ml of supernatant fluid. The mixture was left in the ice bath for 15 minutes after addition of the alcohol and then centrifuged at  $0^{\circ}$  for 20 minutes at 12,500 r p m, the precipitate was discarded and the volume of the supernatant fluid was recorded. An additional 135 ml of alcohol for each 100 ml of this fluid were added under the conditions used above. The addition was made over a 30 minute period and the precipitate was allowed to form for 30 minutes in the ice bath before it was centrifuged at 10,000 r p m and  $0^{\circ}$  for 10 minutes. The supernatant solution was discarded and the precipitate was suspended in 25 ml of cold water, dialyzed for 3 hours with three changes of water, and centrifuged in order to remove insoluble material. The supernatant solution was made  $2 \times 10^{-2}$  M with respect to imidazole buffer of pH 7 and  $2.5 \times 10^{-4}$  M with respect to 3PGA.

*Second Alcohol Fractionation Step VI*—7.5 ml of 95 per cent ethanol per 10 ml of the supernatant fluid from Step V were added, as in the previous step, over a 30 minute period to the solution which was kept in an ice bath during the addition and for 16 hours thereafter. Centrifugation at 10,000 r p m and  $0^{\circ}$  for 10 minutes removed a precipitate which was discarded. The volume of the supernatant solution was noted prior to its return to the ice bath and an additional 5.7 ml of ethanol were added per 10 ml of supernatant solution over a 30 minute interval. A fine white precipitate appeared during addition of the alcohol, and the mixture was centrifuged as before after 30 minutes storage on ice. The precipitate was dissolved in 10 ml of 0.015 M imidazole buffer, pH 7, and any insoluble material was removed by centrifugation<sup>3</sup>.

*Starch Electrophoresis Step VII*—The procedures and apparatus were those described by Paigen (11). The trough of the apparatus was filled with a suspension of 0.05 M imidazole buffer, pH 7, and potato starch. A mixture of starch and mutase from the second alcohol fractionation was added at the origin, and a potential of 300 volts was applied across the 30 cm trough for 20 hours. The trough was then disconnected from the electrode vessels and a strip of Whatman No. 1 filter paper was pressed

<sup>3</sup> Attempts at this stage to purify the enzyme further by fractionation with ammonium sulfate or with zinc chloride plus ammonium sulfate gave a number of active fractions, but none had marked enrichment.

against the starch. The paper removed excess buffer and could be treated with a protein stain, bromophenol blue (12), and used as a guide for section of the starch. The enzyme was found in a 2 cm band which was 2.5 cm from the origin toward the anode.

Enzyme preparations at various stages of purification have been stored frozen for 6 months and at 2–10° for several weeks with little loss in activity, but repeated freezing and thawing led to a rapid decline in activity.

### RESULTS AND DISCUSSION

In twenty preparations of the enzyme, the protein concentration of the initial extract was found to vary from 8 to 20 mg per ml. In extracts with high initial protein, a 10-fold purification was the maximum obtainable.

TABLE I  
*Purification of Mutase*

Step No		Activity, units per ml	Mg. of protein per ml	Specific activity, units per mg of protein	Total units of activity
I	Extraction	150	8	18.7	168,000
II	1st heat denaturation	150	4	37.4	150,000
III	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> pptn	1500	28	53.5	135,000
IV	2nd heat denaturation	900	9.5	95	129,000
V	1st alcohol fractionation	1428	10	143	54,300
VI	2nd " "	8000	15	533	80,000

after Step VI (Table I), while, in preparations in which the protein was initially low, a 30-fold purification was achieved at the same stage. The initial enzymatic activity per ml was the same whether the total protein was high or low. Starch electrophoresis removed two protein impurities and resulted in a further 3-fold enrichment without loss in total activity. The enzyme gave one peak in the ultracentrifuge ( $s_{200}=3.69$ ) which was characteristic of a homogeneous protein with a molecular weight in the region of 50,000 (Fig. 1). However, the preparation still contained two components which could be distinguished when the material was subjected to boundary electrophoresis at pH 8.2.

The Michaelis constant was determined for 3PGA from a Lineweaver-Burk plot (Fig. 2), and a value of  $5.0 \times 10^{-3}$  M was obtained. From the same data the turnover number at enzyme saturation and at pH 7 was calculated to be  $5 \times 10^6$  M per minute. Although many glycolytic enzymes are present in muscle in large amounts, none shows this degree of activity *in vitro*, save that triose phosphate isomerase does approach this activity with a turnover number of  $1 \times 10^6$  M per minute (13). It is of interest

that both of these rapid enzymatic reactions are readily catalyzed by chemical agents. The phosphate migration,  $2\text{PGA} \rightleftharpoons 3\text{PGA}$ , is catalyzed by acid and the triose isomerization by alkali.

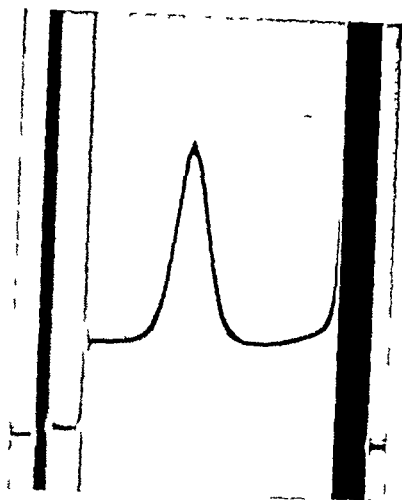


FIG 1 The ultracentrifugal pattern of phosphorylglyceric acid mutase. Time, 80 minutes, speed, 59,780 r p m

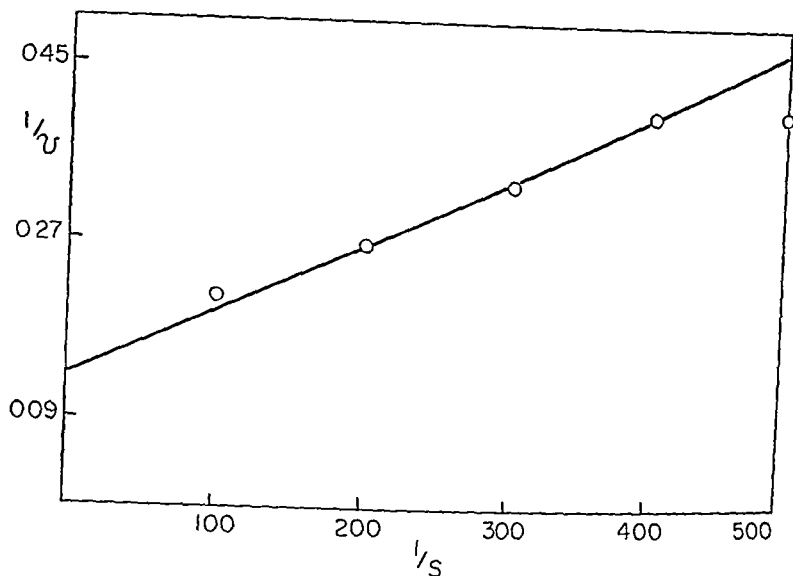


FIG 2 The relationship between substrate concentration and enzymic activity as determined by Assay A,  $37^\circ$  and pH 7.0 in 0.03 M imidazole buffer

The enzyme exhibited optimal activity at pH 7.0 in imidazole buffer. The decrease in activity on either side of this optimum can be ascribed to shifts in the ionic species of the substrate or enzyme or both rather than to enzyme denaturation, for the enzyme could be maintained at pH value

from 3.4 to 10 for 15 minutes at 0° with no effect on subsequent enzymatic activity (assays were carried out at pH 7), (Fig 3)

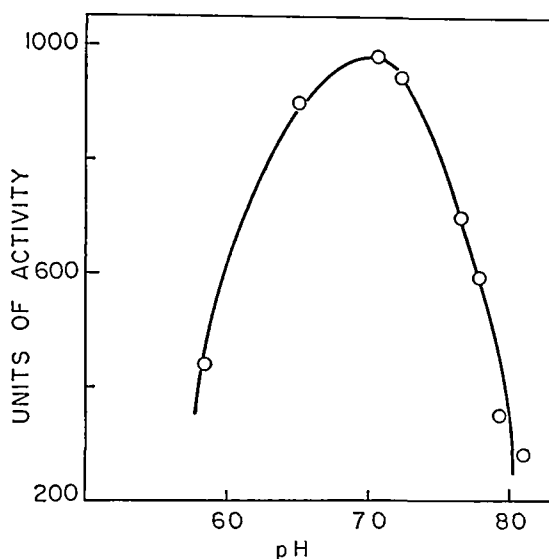


FIG 3 Optimal pH of phosphorylglyceric acid mutase Assays were made in 0.10 M imidazole buffer by Assay A (Assay by the polarimetric method, although less sensitive, gave the same optimum)

TABLE II  
*Fluoride Inhibition of Enzyme Activity*

Other agents	Concentration of fluoride	Inhibition
	<i>M</i>	<i>per cent</i>
2PGA → 3PGA		
None	$1 \times 10^{-2}$	100
"	$5 \times 10^{-3}$	90
"	$1 \times 10^{-3}$	0
$5 \times 10^{-3}$ M $Mg^{++}$	$5 \times 10^{-3}$	90
$1 \times 10^{-3}$ " Versene	$1 \times 10^{-2}$	85
3PGA → 2PGA		
None	$1 \times 10^{-2}$	90
"	$1 \times 10^{-3}$	0
$5 \times 10^{-3}$ M $Mg^{++}$	$1 \times 10^{-2}$	90
$5 \times 10^{-3}$ " "	$1 \times 10^{-3}$	0

In contrast to phosphoglucomutase and many other enzymes that catalyze phosphate transfer reactions, no metal ion requirement could be demonstrated for phosphorylglyceric acid mutase. Dialysis against Versene or Dowex 50 ( $H^+$ ) did not reduce enzymatic activity, and addition

of metals to the dialyzed enzyme had no activating effect. Direct addition of Versene to the incubation mixture of Assay B to give a final concentration of  $3 \times 10^{-3}$  M did not alter enzymatic activity, hence, it would appear that, if metals do play a role in this reaction, they must be tightly bound to the protein. However, the purified enzyme was strongly inhibited by fluoride ions at  $10^{-2}$  M (Table II). The concentration of fluoride which produced inhibition was independent of added metal ions, and no evidence

TABLE III  
*Effect of Metal Ions and Sulfhydryl Inhibitors on Phosphorylglyceric Acid Mutase*

Inhibitors were added directly to the enzyme assay mixture unless otherwise indicated

Inhibitor	Concentration	Inhibition
	<i>M</i>	<i>per cent</i>
2PGA $\rightarrow$ 3PGA		
Hg <sup>++</sup>	$1 \times 10^{-4}$	100
Ag <sup>+</sup>	$1 \times 10^{-4}$	85
Ba <sup>++</sup>	$1 \times 10^{-4}$	0
Mg <sup>++</sup>	$1 \times 10^{-3}$	0
Cu <sup>+++</sup>	$2.5 \times 10^{-3}$	90
<i>p</i> -Chloromercuribenzoate*	$5 \times 10^{-5}$	100
Iodoacetate*	$1 \times 10^{-3}$	70
3PGA $\rightarrow$ 2PGA		
Hg <sup>++</sup>	$1 \times 10^{-4}$	70
Ag <sup>+</sup>	$1 \times 10^{-4}$	70
Pb <sup>++</sup>	$1 \times 10^{-4}$	0
Zn <sup>++</sup>	$1 \times 10^{-4}$	0
Cu <sup>++</sup>	$1 \times 10^{-4}$	0
<i>p</i> -Chloromercuribenzoate*	$1 \times 10^{-4}$	100
Iodoacetate*	$1 \times 10^{-3}$	60

\* Preincubation with the enzyme for 10 minutes at 37° in the absence of substrate

was found for a metal-substrate-fluoride inhibitor complex such as those found for enolase (8) and phosphoglucumutase (14). The high fluoride concentration might suggest a non-specific inhibition, but a simple salt effect is excluded since chloride or cyanide at equal or greater concentrations did not inhibit. It would appear that fluoride acts in some specific but as yet unknown manner. An apparently similar type of inhibition was reported by Nikiforuk and Colowick (15) for adenylic acid deaminase.

Of the metal ions tested by direct addition to the incubation mixture of Assay B, silver and mercury ions at  $10^{-4}$  M inhibited strongly. This suggested that there might be sulfhydryl groups at or near the enzymatically active site. It had been previously reported (3) that iodoacetate

had no effect on the activity of phosphorylglyceric acid mutase in crude extracts, but we have found that incubation of the purified enzyme with  $10^{-3}$  M iodoacetate or  $10^{-5}$  M *p*-chloromercuribenzoate before addition of substrate effectively inhibited enzymatic activity. Cupric salts, which are known reagents for sulfhydryl groups, did not inhibit when added to the enzyme in the presence of substrate but did inhibit when the enzyme was previously incubated with the cupric salts in the absence of substrate. These results indicate a protection of the active site by the substrate. As both 2PGA and 3PGA exhibit this protective influence, it will remain high throughout the reaction (Table III).

TABLE IV

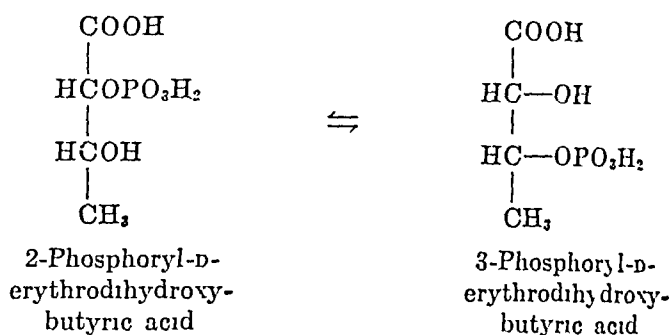
*Inhibition of Mutase by Structurally Related Compounds*

The reaction measured was 2PGA  $\rightarrow$  3PGA

Compounds	(Inhibitor)/(2PGA)	Inhibition
		<i>per cent</i>
CH <sub>2</sub> CHO <i>CHOPO<sub>2</sub>H<sub>2</sub> COOH</i> (D-)	3.7	35
CH <sub>2</sub> CHOPO <sub>2</sub> H <sub>2</sub> <i>CHOH COOH</i> (D-)	3.0	35
CH <sub>2</sub> <i>CHOPO<sub>2</sub>H<sub>2</sub> COOH</i> (D-)	3.7	65
CH <sub>2</sub> OPO <sub>2</sub> H <sub>2</sub> CH <sub>2</sub> COOH	4.0	0
CH <sub>2</sub> <i>CHOH COOH</i> (D-)	4.0	30
CH <sub>2</sub> OH <i>CHOH COOH</i> (D-)	4.0	30
CH <sub>2</sub> OPO <sub>2</sub> H <sub>2</sub> CHOH CHO (D-)	4.0	(15)*
CH <sub>2</sub> CH <sub>2</sub> COOH	4.0	0

\* Value of questionable significance as an inhibition

The inhibitory activity of several compounds structurally related to the substrates was tested by Assay B, and the results are summarized in Table IV. For the inhibition of the reaction 2PGA  $\rightarrow$  3PGA, the inhibitor would appear to need a terminal carboxyl group and a phosphorylated or free hydroxyl group on the  $\alpha$ -carbon. These structures are italicized in Table IV. The nature of the substituents on the  $\beta$ -carbon did not appear to be critical for this inhibition. Further work with structurally related compounds will be required to reveal the importance of the stereochemical configuration of the inhibitor and of the structural requirements for inhibition of the reverse reaction. In addition to their inhibitory action (Table IV), the phosphoryl-D-erythrodihydroxybutyric acids could serve as substrates for the mutase, although the reaction was several thousand-fold slower than that for the normal phosphorylglyceric acid substrates. The equilibrium constant for the reaction of the phosphorylerythrodihydroxybutyric acids was 3.9 at pH 6.8 and 37°.



Since the purified mutase was free from enolase, a direct determination of the equilibrium constant for the reaction  $2\text{PGA} \rightleftharpoons 3\text{PGA}$  was possible. Meyerhof and Kiessling (1) had measured this equilibrium in crude rabbit muscle extracts and had found a value of 6. From the data of Warburg and Christian (8) for the equilibrium of the enolase-catalyzed reaction and the over-all equilibrium of the coupled enolase- and mutase-catalyzed reactions, one may calculate an equilibrium constant of 3 for the reaction  $2\text{PGA} \rightleftharpoons 3\text{PGA}$ . Ballou and Fischer (6) reported a value of 4.1 for the acid-catalyzed equilibrium of  $3\text{PGA} \rightleftharpoons 2\text{PGA}$  in 1 N HCl. The enzyme-catalyzed equilibrium was measured at pH 6.8 and  $37^\circ$  by Assay B with both  $2\text{PGA}$  and  $3\text{PGA}$  as the starting component. The results are shown in Fig. 4 and the equilibrium constant,  $K = 3\text{PGA} / 2\text{PGA}$ , was observed to be 5.0.

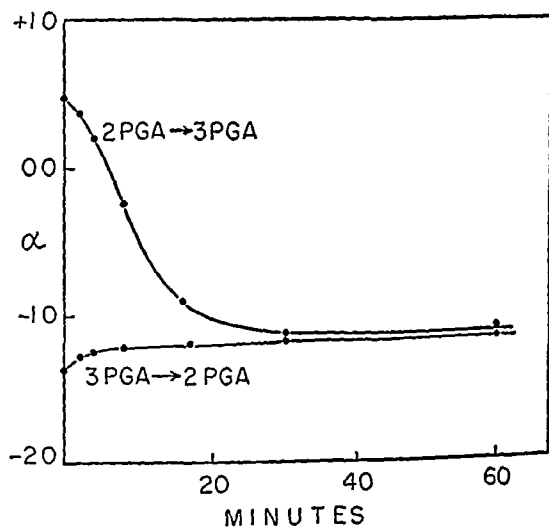


FIG. 4 The change in optical rotation when either  $2\text{PGA}$  or  $3\text{PGA}$  was incubated with purified mutase. Enzyme incubation was made by Assay B at pH 6.8 and  $37^\circ$ , optical rotations were made in 8 per cent ammonium molybdate.  $[\alpha]_D^{25}$  is  $+5^\circ$  for  $2\text{PGA}$  and  $-74^\circ$  for  $3\text{PGA}$  (6).

## SUMMARY

Phosphorylglyceric acid mutase was extensively purified from rabbit skeletal muscle. The enzyme was found to have a high turnover number and a pH optimum at 7.0. No metal ion requirement for enzyme activity could be demonstrated but the enzyme was inhibited by  $10^{-2}$  M fluoride. The mutase was inhibited also by heavy metal ions and sulfhydryl reagents. A number of compounds related to phosphorylglyceric acid were tested as inhibitors, and certain structural features were concluded to be essential for such inhibition. The equilibrium constant for the enzymatic interconversion of the two phosphorylglyceric acids was 5.0.

We wish to acknowledge gifts of synthetic 2-phosphoryl-D-glyceric acid and 3-phosphoryl-D-glyceric acid, and the phosphorylethyrodihydroxybutyric acids from Dr. C. E. Ballou and the gift of crystalline enolase from Mr. Finn Wold. The ultracentrifuge and electrophoretic analyses were conducted in the laboratory of Dr. H. K. Schachman.

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# THE SYNTHESIS OF L-ASCORBIC ACID IN THE RAT FROM D-GLUCURONOLACTONE AND L-GULONOLACTONE\*

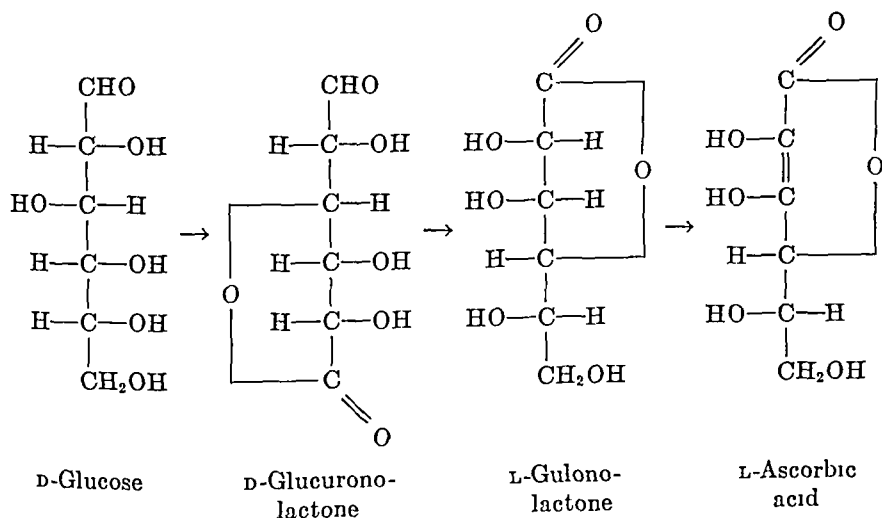
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Previous studies have shown that the intact carbon chain of glucose is utilized for the synthesis of L-ascorbic acid in the rat (1-3). Evidence for this precursor relationship came from experiments in which the administration of carbon 1- and carbon 6-labeled glucose tracers resulted in the urinary excretion of L-ascorbic acid labeled predominantly in carbon 6 and carbon 1, respectively. Although these earlier studies were carried out in rats receiving Chloretone to increase their normal rate of L-ascorbic acid biosynthesis (4), recent results have shown that glucose is also a precursor of L-ascorbic acid in rats not treated with this drug (5).

Two possible pathways for the biosynthesis of L-ascorbic acid from glucose in rats have been investigated. One, involving L-sorbose as an intermediate, was ruled out in a previous isotopic study (6). The other pathway involving D-glucuronolactone and L-gulonolactone as intermediates is shown in the accompanying scheme.



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Some evidence for this latter scheme has been presented previously. Isherwood and coworkers (7) reported that the administration of D-glucuronolactone and L-gulonolactone to rats produced an increase in the urinary excretion of L-ascorbic acid. In addition, Horowitz and King (8) found that the administration of uniformly labeled D-glucuronolactone to Chloretone-treated rats resulted in the excretion of uniformly labeled L-ascorbic acid.

In the present investigation further evidence for this pathway of L-ascorbic acid biosynthesis was obtained by demonstrating that normal and Chloretone-treated rats are able to convert carboxyl-labeled D-glucuronolactone and L-gulonolactone to carboxyl-labeled L-ascorbic acid.<sup>1</sup> Results were also obtained which indicate that neither compound is converted to L-ascorbic acid in guinea pigs. Incidental to this study, observations were made showing that L-gulonolactone is appreciably oxidized to CO<sub>2</sub> in guinea pigs and rats.

#### EXPERIMENTAL

*Radioactive Compounds*—D-Glucuronolactone-6-C<sup>14</sup>, sodium D-glucuronate-6-C<sup>14</sup>, and D-glucose-1-C<sup>14</sup> were obtained from the National Bureau of Standards, Washington, D. C., and the compounds had specific activities of 0.68, 0.65, and 1.0  $\mu$ c per mg, respectively. L-Gulonolactone-1-C<sup>14</sup> was synthesized by reducing sodium D-glucuronate-6-C<sup>14</sup> with sodium borohydride (10).<sup>2</sup> The L-gulonolactone-1-C<sup>14</sup> had a specific activity of 0.17  $\mu$ c per mg after recrystallization from glacial acetic acid. Material prepared in a non-radioactive trial synthesis had a melting point of 184–185° and an optical rotation of  $[\alpha]_D^{20} +53.6^\circ$  (c 0.3, water) (10). Its elemental analysis was as follows:

C<sub>6</sub>H<sub>10</sub>O<sub>6</sub>. Calculated, C 40.4, H 5.66, found, C 40.2, H 5.84.

L-Gulonic acid-1-C<sup>14</sup> was prepared by treating L-gulonolactone-1-C<sup>14</sup> at 50° with a stoichiometric amount of NaOH in aqueous solution.

*Experimental Animals*—Male albino rats of the Wistar strain, weighing from 270 to 310 gm, were maintained on a basal diet of evaporated milk for at least 10 days before each experiment. In the experiments on the effect of Chloretone, 45 mg of the drug were administered daily for a period of at least 4 days prior to the injection of the labeled compound. The Chloretone was given in a single dose by stomach tube as a homogenate in 1 ml of evaporated milk. Male guinea pigs, weighing 250 to 300 gm, were maintained on a vitamin C-free diet supplemented daily with 5 mg

<sup>1</sup> A preliminary report of this work has been presented previously (9).

<sup>2</sup> Some details of this synthesis were kindly supplied by Dr. H. S. Isbell of the National Bureau of Standards, Washington, D. C.

of L-ascorbic acid orally for 7 days prior to each experiment. The labeled compounds employed in the various experiments were dissolved in 1 ml. of water, and they were administered by intraperitoneal injection.

*Conversion to L-Ascorbic Acid*—The conversion of the various precursors to L-ascorbic acid in normal rats and guinea pigs was determined by estimating the amount of  $C^{14}$ -L-ascorbic acid present in the animal 24 hours after administration of the labeled compounds. This was done as follows. The animals were sacrificed, and then livers, adrenals, spleens, testes, and kidneys were removed. The pooled sample of the various tissues of each animal was homogenized in the cold with about four times its weight of 5 per cent trichloroacetic acid, and the protein residue was removed by centrifugation. L-Ascorbic acid present in the supernatant fluid was determined by titration of an aliquot with indophenol dye (11). The L-ascorbic acid in the remaining supernatant fluid was isolated by an ion exchange method (4, 12) after the addition of a weighed quantity of carrier L-ascorbic acid (100 to 200 mg). The specific activity of the L-ascorbic acid present originally in the tissue sample was calculated from the specific activity of the isolated L-ascorbic acid and the amount of L-ascorbic acid present before addition of carrier. The per cent incorporation of the various labeled precursors into L-ascorbic acid was obtained by multiplying the calculated specific activity (expressed as per cent of dose per mg) by the body pool of L-ascorbic acid, 10 mg per 100 gm body weight for rats (4) and 5.4 mg per 100 gm body weight for guinea pigs<sup>3</sup>. The results of these experiments give minimal values for the incorporation of the various labeled compounds into L-ascorbic acid, since no correction is made for the amount of labeled L-ascorbic acid metabolized and excreted in the urine during the 24 hour period after their administration. This correction would be relatively small, however, since L-ascorbic acid is slowly metabolized and excreted by normal rats and guinea pigs with a half life of about 3 days in both species (4, 13).

The conversion of the various labeled compounds to L-ascorbic acid in Chloretone-treated rats was determined by measuring the incorporation of  $C^{14}$  into urinary L-ascorbic acid collected during a 24 hour period after administration of the labeled compounds (1, 6).

*Degradation Procedure*—The  $C^{14}$  in carbon 1 of L-ascorbic acid was obtained as  $CO_2$  by decarboxylation with hot mineral acid (1).

*Measurement of Radioactivity*—The method for collection and preparation of samples and their assay for  $C^{14}$  were described previously (6). The radioactive purity of the L-ascorbic acid isolated from urine and tissues was established by finding constant specific activity of the L-ascorbic acid

<sup>3</sup> Dayton, P. G., and Burns, J. J., to be published.

and its 2,4-dinitrophenylosazone derivative after successive crystallizations (1, 6)

### Results

The incorporation of  $C^{14}$  into body L-ascorbic acid of normal rats was measured 24 hours after the administration of D-glucose-1- $C^{14}$ , D-glucuronolactone-6- $C^{14}$ , L-gulonolactone-1- $C^{14}$ , sodium D-glucuronate 6  $C^{14}$ , and sodium L-gulonate-1- $C^{14}$  (Table I). It will be noted that the per cent incorporation of D-glucuronolactone-6- $C^{14}$  and L-gulonolactone-1- $C^{14}$  averaged 2.2 and 8.1 per cent, respectively, compared to 0.045 per cent for

TABLE I  
*Incorporation of  $C^{14}$  into Body L-Ascorbic Acid 24 Hours after Administration of Labeled Compounds to Normal Rats*

Experiment No	Compound	Dose of labeled compound	Per cent of d l-L-ascorbic acid
		mg	
R-84	D-Glucose-1- $C^{14}$	35.0	0.033
R-85	"	35.0	0.033
R-20	D-Glucuronolactone-6- $C^{14}$	8.2	2.3
R-22	"	8.2	2.0
R-31	L-Gulonolactone-1- $C^{14}$	11.9	9.1
R-32	"	11.9	7.2
R-53	D-Glucuronate-6- $C^{14}$ †	5.1	<0.2
R-78	"	5.1	<0.2
R-49	L-Gulonate-1- $C^{14}$ †	12.2	<0.3
R-52	"	12.2	<0.1

\* For method of calculation see the experimental section

† Sodium salt

D-glucose-1- $C^{14}$ . No  $C^{14}$  was detected in L-ascorbic acid after administration of D-glucuronate-6- $C^{14}$  and L-gulonate-1- $C^{14}$ , indicating that the lactone structures are apparently required for L-ascorbic acid biosynthesis.

The incorporation of  $C^{14}$  into urinary L-ascorbic acid during 24 hours following administration of D-glucose-1- $C^{14}$ , D-glucuronolactone 6  $C^{14}$ , and L-gulonolactone-1- $C^{14}$  to Chlorotone-treated rats was measured (Table II). It will be noted that the incorporation of D-glucuronolactone 6  $C^{14}$  and L-gulonolactone-1- $C^{14}$  into urinary L-ascorbic acid averaged 1.2 and 3.6 per cent, respectively, compared to 0.45 per cent for glucose-1- $C^{14}$ . However, these are minimal values for the incorporation of these precursors into L-ascorbic acid, since about one-half the total L-ascorbic acid synthesized daily by Chlorotone-treated rats is not excreted in the urine but is further metabolized (4). The actual values for the incorporation of the various

compounds into L-ascorbic acid are, therefore, about twice those reported in Table II, that is 0.9, 2.4, and 7.2 per cent for D-glucose, D-glucuronolactone, and L-gulonolactone, respectively. These values for the incorpora-

TABLE II  
*Incorporation of C<sup>14</sup> into Urinary L-Ascorbic Acid during 24 Hours after Administration of Various Labeled Compounds to Chloretone-Treated Rats*

Experiment No	Compound *	Dose of labeled compound	Per cent of dose in L-ascorbic acid
		mg	
R-1*	D-Glucose-1-C <sup>14</sup>	10.0	0.50
R-2*	"	10.0	0.40
R-5A	D-Glucuronolactone-6-C <sup>14</sup>	16.1	1.1
R-5B	"	16.1	1.3
R-42	L-Gulonolactone-1-C <sup>14</sup>	6.4	3.9
R-43	"	6.4	3.8
R-96	"	6.4	3.1

\* Previously published experiments (5)

TABLE III  
*Distribution of C<sup>14</sup> in L-Ascorbic Acid after Administration of Various Labeled Compounds to Normal and Chloretone-Treated Rats*

Experiment No	Compound	Per cent of total C <sup>14</sup> in L-ascorbic acid in carbon 1
Normal		
R-20	D-Glucuronolactone-6-C <sup>14</sup>	85
R-22	"	83
R-31	L-Gulonolactone-1-C <sup>14</sup>	87
R-32	"	87
Chloretone-treated		
R-5A	D-Glucuronolactone-6-C <sup>14</sup>	88
R-5B	"	92
R-42	L-Gulonolactone-1-C <sup>14</sup>	84
R-96	"	85

tion of labeled D-glucuronolactone and L-gulonolactone to L-ascorbic acid in the Chloretone-treated rat are similar to those obtained for the incorporation of these lactones into L-ascorbic acid in the non-drug-treated animal (Table I). However, it should be noted that there is a marked difference in the incorporation of labeled glucose into L-ascorbic acid in

normal and Chloretone-treated rats which is a reflection of the increased amounts of L-ascorbic acid synthesized in the drug-treated animal (4)

The distribution of  $C^{14}$  in the L-ascorbic acid synthesized after administration of D-glucuronolactone-6- $C^{14}$  and L-gulonolactone-1- $C^{14}$  to normal and Chloretone-treated rats was measured (Table III). The L-ascorbic acid isolated in the various experiments was labeled predominantly in carbon 1, indicating that D-glucuronolactone and L-gulonolactone are

TABLE IV  
 *$C^{14}$  in Body L-Ascorbic Acid 24 Hours after Administration of Labeled Compounds to Guinea Pigs*

Experiment No	Compound	Dose of labeled compound	Per cent of total L-ascorbic acid $C^{14}$
		mg	
G-25A	D-Glucuronolactone-6- $C^{14}$	4.9	<0.1
G-25B	"	4.9	<0.1
G-36	L-Gulonolactone-1- $C^{14}$	11.7	<0.2
G-37	"	11.7	<0.2

\* For method of calculation see the experimental section

TABLE V  
 *$C^{14}$  in Expired  $CO_2$  and Urine during 24 Hour Period after Intraperitoneal Administration of L-Gulonolactone-1- $C^{14}$  to Guinea Pigs and Rats*

Experiment No	Species	Per cent of dose in	
		$CO_2$	Urine
R-31	Rat	69	29
R-32	"	68	22
G-36	Guinea pig	55	30
G-37	" "	67	25

converted to L-ascorbic acid without appreciable fragmentation of their carbon chains

The incorporation of  $C^{14}$  into body L-ascorbic acid of guinea pigs was measured 24 hours after the administration of D-glucuronolactone-6- $C^{14}$  and L-gulonolactone-1- $C^{14}$  (Table IV). It will be noted that no detectable incorporation of either compound was observed in guinea pig, value being less than one-twentieth those in rats.

The excretion of  $C^{14}$  in respiratory  $CO_2$  and urine was compared following administration of L-gulonolactone-1- $C^{14}$  to guinea pigs and rats (Table V). L-Gulonolactone is appreciably oxidized to  $CO_2$  in guinea pigs and rats; most of the  $C^{14}$  in  $CO_2$  was recovered within 5 hours after the dose. In

contrast, sodium gulonate-1-C<sup>14</sup> is oxidized to CO<sub>2</sub> to a considerably lesser extent than L-gulonolactone-1-C<sup>14</sup> as shown by the results of the following experiment. Two rats each received a 12 mg intraperitoneal dose of sodium L-gulonate-1-C<sup>14</sup> and the amounts of C<sup>14</sup> in respiratory CO<sub>2</sub> and urine were determined over a 24 hour period. Essentially all the injected C<sup>14</sup> was found in the urine, less than 10 per cent of the dose being present in the respiratory CO<sub>2</sub>.

#### DISCUSSION

The results of this investigation are in agreement with a pathway of L-ascorbic acid biosynthesis from D-glucose via D-glucuronolactone and L-gulonolactone in both normal and Chloretone-treated rats. For instance, finding that carboxyl-labeled D-glucuronolactone and L-gulonolactone are converted to L-ascorbic acid labeled chiefly in carbon 1 indicates that the carbon chain of these lactones is converted without fragmentation to L-ascorbic acid. Since no information is available on the turnover and pool sizes of D-glucuronolactone and L-gulonolactone, it is not possible at the present time to estimate how much of the total L-ascorbic acid synthesized each day in normal and Chloretone-treated rats originates via these compounds.

The most likely intermediate involved in the conversion of L-gulonolactone to L-ascorbic acid is either 2-keto- or 3-keto-L-gulonolactone. Since these compounds undergo spontaneous enolization to L-ascorbic acid (14), their role in the biosynthesis of L-ascorbic acid in rats was not evaluated in this study. Experiments in rats with C<sup>14</sup>-labeled 2-keto-L-gulonic acid indicate, however, that this acid is not an intermediate in the conversion of L-gulonolactone to L-ascorbic acid.<sup>3</sup>

The results of this investigation, indicating that D-glucuronolactone and L-gulonolactone are not converted to L-ascorbic acid in the guinea pig, point out the possible missing biochemical reaction in this species and perhaps in man needed for the synthesis of L-ascorbic acid. A more definitive conclusion concerning the specific biochemical reaction which is missing will be possible when information is available on whether or not D-glucuronolactone can be converted to L-gulonolactone in the guinea pig.

It has been known for considerable time that certain drugs such as Chloretone and various barbiturates when administered to rats markedly increase the urinary excretion of L-ascorbic acid (15). Results of a previous turnover rate study showed that this increase in excretion of L-ascorbic acid results from an actual acceleration in its rate of synthesis (4). The results of the present study suggest that the primary effect of Chloretone is to increase the synthesis of D-glucuronolactone which is then utilized for the synthesis of L-ascorbic acid. Support for this interpretation comes



from the observation that Chloretone administration to rats produces a marked increase in the excretion of both L-ascorbic acid and D-glucuronic acid (16). In the guinea pig, however, Chloretone administration results only in an increase in the excretion of D-glucuronic acid,<sup>4</sup> since this species is unable to synthesize L-ascorbic acid. At this time no conclusion can be drawn on the actual mechanism by which a drug, such as Chloretone, accelerates the synthesis of D-glucuronolactone. However, the ability of a drug to be conjugated as a glucuronide is apparently not required, since barbitol, which is excreted completely unchanged in the urine, markedly increases the urinary excretion of both D-glucuronic acid and L-ascorbic acid by rats and of D-glucuronic acid by guinea pigs.<sup>4</sup>

#### SUMMARY

Carboxyl-labeled D-glucuronolactone and L-gulonolactone are converted in normal and Chloretone-treated rats to carboxyl-labeled L-ascorbic acid, indicating that the intact carbon chain of these lactones is utilized for the biosynthesis of L-ascorbic acid. Carboxyl-labeled D-glucuronic acid and L-gulonic acid, however, are not converted to L-ascorbic acid in the rat, indicating the importance of the lactone structure in the biosynthesis of L-ascorbic acid.

No conversion of L-gulonolactone and D-glucuronolactone to L-ascorbic acid was detected in the guinea pig, an indication of the possible missing biochemical reaction in this species needed for the synthesis of L-ascorbic acid.

Carboxyl-labeled L-gulonolactone was found to be extensively oxidized to CO<sub>2</sub> in both rats and guinea pigs. However, no appreciable oxidation of carboxyl-labeled sodium L-gulonate was observed.

The authors of the paper gratefully acknowledge the assistance of Dr. Erwin H. Mosbach in carrying out the synthesis of the labeled L-gulonolactone.

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# THE CONVERSION OF VARIOUS CARBOHYDRATES TO 5-DEHYDROSHIKIMIC ACID BY BACTERIAL EXTRACTS\*

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Nutritional studies with bacterial mutants have shown that 5-dehydroquinic acid (DHQ) (1), 5-dehydroshikimic acid (DHS) (2), and shikimic acid (SA) (3) are successive intermediates in the biosynthesis of several aromatic amino acids and vitamins.

In order to reveal earlier reactions in this pathway studies were undertaken on the synthesis of SA from variously labeled glucose by intact cells. The results indicated that SA arose via a triose and a tetrose (4). Meanwhile, for detailed analysis of the pathway a search had been undertaken for a preparation that would carry out these early reactions unhampered by permeability barriers. Such a preparation became available through the finding, to be described in the present paper, that some extracts of a mutant blocked after DHS can form this compound from various phosphorylated carbohydrates. Part of this work has been published in preliminary form (5).

## EXPERIMENTAL

*Preparation of Bacterial Extract*—The reactions sought could have been detected through formation of DHQ, the earliest member of this sequence that can be readily assayed (1, 6). However, since the DHS/DHQ equilibrium constant of 15 (7) might be expected to promote the preceding

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reactions, a mutant blocked immediately after DHS was used rather than one blocked between DHQ and DHS

The organism, *Escherichia coli* mutant 83-2, was grown for 18 hours with aeration at 37° in 400 ml of minimal medium A (8) supplemented with 0.2 per cent Difco yeast extract and 0.2 per cent casein hydrolysate (Sheffield NZ-Case). The cells (approximately 4 gm wet weight) were harvested by centrifugation, washed with cold water, suspended in 20 ml of cold potassium phosphate buffer (M/30, pH 7.4), and disrupted by means of sonic oscillation for 30 minutes in a cooled 9 kc Raytheon magnetostriiction oscillator, model S-102A. Centrifugation at  $12,000 \times g$  for 30 minutes at 0° yielded a clear greenish yellow supernatant solution, which was diluted with the same buffer to a final protein concentration of 20 mg per ml. The extract was used without further purification. At -15° it retained adequate activity for at least 6 months.

**Assay of DHS**—Samples of the incubation mixture, taken at zero time and after the stated intervals, were brought to pH 1 with 6 N HCl and the precipitated protein was removed by centrifugation. 0.1 and 0.3 ml portions of the supernatant solution were pasteurized (60°, 15 minutes) and were assayed turbidimetrically with a quintuple aromatic auxotroph, *Aerobacter aerogenes* strain A170-143S1 (6). This mutant responds equally well to DHQ, DHS, or SA, the last compound was used as a standard. The assay medium (final volume 10 ml) was minimal medium A supplemented with 20  $\gamma$  each of L-tyrosine and L-phenylalanine per ml. This supplement markedly increases the sensitivity of the assay by sparing a large part of the DHS requirement (6).

**Identification of Reaction Product As DHS**—Under the assay conditions used the aromatic vitamins present in the crude extracts would allow a response not only to DHS or SA but also to tryptophan or its known specific precursors, indole and anthranilic acid. Accordingly, a number of the samples that supported growth of the assay organism were also tested with an *E. coli* mutant, 121-35, that responds to tryptophan or its precursors but not to DHS. No growth of this mutant could be detected.

The material that was active in the assay with mutant A170-143S1 was equally active for *E. coli* mutant 83-1, which can respond to DHS or SA but not to DHQ (6), but there was no activity for *E. coli* mutant 156-53D2 which can respond to SA but not to DHS (9). Furthermore, paper chromatograms, developed with butanol-formic acid-water (20:1:4) and tested by bioautography with strain A170-143S1, yielded the same  $R_F$  value (0.12 to 0.45) for the reaction product, for authentic DHS (2), and for a mixture of the two. Finally, the active material was converted on appropriate treatment to protocatechuic acid (see below) and to SA (10), which were isolated.

### Results

*Formation of DHS from Glucose-6-phosphate (G-6-P), Incorporation of Labeled G-6-P*—As shown in Table I, on incubating the bacterial extract for 4 hours with glucose as substrate the yield of DHS was negligible, but it became significant when adenosine triphosphate (ATP) was also added. The yield was increased 3-fold by preincubating the glucose and ATP with hexokinase before adding the extract or by using commercial glucose-6-phosphate (G-6-P) as substrate.

TABLE I

*Conversion of Glucose-6-phosphate to 5-Dehydroshikimic Acid*

The reaction mixture contained, per ml, 5  $\mu$ moles of  $MgCl_2$ , 50  $\mu$ moles of potassium phosphate buffer (pH 7.4), and 0.1 ml of bacterial extract (20 mg of protein per ml). Incubated at 37° for 4 hours. See "Experimental" for further details.

Substrate	Concentration	Conversion to DHS*
	$\mu$ moles per ml	per cent
Glucose	10	0.4
ATP	6	0
Glucose + ATP	10 + 6	1.4
G 6 P (from hexokinase reaction)†	5	4.0
" (commercial)	5	4.0

\* Average values of several experiments. The yields were calculated on the basis of a theoretical formation of 6 moles of DHS from 7 moles of hexose.

† The hexokinase reaction mixture contained, per ml, 100  $\mu$ moles of tris(hydroxymethyl)aminomethane buffer, pH 7.4, 10  $\mu$ moles of  $MgCl_2$ , 10  $\mu$ moles of KCl, 6  $\mu$ moles of glucose, 6  $\mu$ moles of ATP, and 0.2 ml of hexokinase solution. After incubation for 1 hour at 37° 83 per cent of the glucose had been converted to G-6-P, as shown by determination of the decrease of readily hydrolyzable organic phosphate (11) compared with a tube without added glucose. Further incubation yielded no significant increase in G-6-P. Such a 1 hour hexokinase reaction mixture was used without further treatment as substrate for DHS formation.

The yield of DHS from G-6-P was found to be maximal (about 5 per cent) after 4 hours of incubation (*cf* Table III below), the rest of the G-6-P possibly being destroyed through competing reactions. Because of the small yield it was not certain whether G-6-P was incorporated into DHS or was used merely as an energy source to convert precursors present in the crude extract. To settle this point labeled G-6-P was prepared from 100  $\mu$ moles of uniformly  $C^{14}$ -labeled glucose by incubating with ATP and hexokinase as in Table I. Bacterial extract (1 ml) was then added, after 4 hours the mixture (10 ml) was adjusted to pH 1 with 6 N HCl and precipitated protein was removed. A small sample was assayed for DHS microbiologically. To the remainder 30 mg of carrier protocatechuic acid

were added and the DHS was converted to protocathechuic acid by adding 0.4 ml of concentrated HCl and boiling for 1 minute (2). The protocathechuic acid was isolated by ether extraction, followed by recrystallization.

TABLE II  
*Conversion of Glucose-C<sup>14</sup> to 5-Dehydroshikimic Acid*

Uniformly labeled glucose-C<sup>14</sup> was converted successively to G 6 P, DHS, and protocathechuic acid as described under "Results."

Compound	Activity of carbon*	Protocatechuic acid
		Glucose activity
Glucose	$c p m \times 10^{-4}$	per cent
Protocatechuic acid (Experiment 1)	2.55	
" " ‡ ( " " 2)	1.47†	65
	1.83†	72

\* Counts per minute at "infinite" thickness under standard conditions divided by the fraction of carbon in the compound.

† Observed activity corrected for dilution with carrier protocathechuic acid.

‡ BaCO<sub>3</sub> obtained by decarboxylation of this sample with Cu powder and quinoline (4) had an activity of  $1.57 \times 10^6$  c p m, indicating that the DHS was essentially uniformly labeled.

TABLE III  
*Synthesis of 5-Dehydroshikimic Acid from Phosphorylated Carbohydrates*  
Reaction mixture as in Table I. Substrate concentration 5  $\mu$ moles per ml.

Substrate	Conversion to DHS*		
	2 hrs	4 hrs	6 hrs
	per cent	per cent	per cent
Glucose-6-phosphate	3.8	5.4	5.8
Glucose-1-phosphate	4.0		
Fructose-6-phosphate	4.6		
Fructose-1,6-diphosphate	4.0	5.6	6.0
Ribose-5-phosphate	2.4	4.0	
6-Phosphogluconic acid	0	0	

\* Average values of several experiments.

from H<sub>2</sub>O. The results obtained in two different experiments (Table II) indicated that 60 to 70 per cent of the carbon of the DHS had been derived from the labeled G-6-P. This value might be low, owing to errors in the microbiological determination of DHS and to possible failure of the treatment to convert DHS quantitatively to protocathechuic acid.

*Conversion of Other Phosphorylated Carbohydrates to DHS—Table III*

summarizes the results obtained with several substrates. The other hexose phosphates tested were as active as G-6-P, and ribose-5-phosphate was nearly as active. 6-Phosphogluconic acid was inactive, presumably owing to the fact that *E. coli* extracts contain very little triphosphopyridine nucleotide (TPN) (12), which is needed for the conversion of this compound to pentose phosphate.

*Conversion of Sedoheptulose-7-phosphate to DHS*—The conversion of glucose to DHS presumably requires at some stage a 7-carbon, open chain intermediate. Sedoheptulose-7-phosphate, an intermediate of the pentose phosphate pathway (13-15), was therefore tested. After 2 hours the

TABLE IV

*Synthesis of 5-Dehydroshikimic Acid from Sedoheptulose-7-phosphate*

Reaction mixture as in Table I

Substrate	Concentration	Conversion to DHS*	
		2 hrs	4 hrs
	<i>μmoles per ml</i>	<i>per cent</i>	<i>per cent</i>
Fructose-1,6-diphosphate	5	3.2	4.6
	2.5	3.2	3.6
Sedoheptulose-7-phosphate	5	1.0	3.8
	2.5	1.5	4.5
Fructose-1,6-diphosphate + sedoheptulose-7-phosphate	2.5 + 2.5	5.0	7.0

\* Average values of several experiments

conversion of this compound was slight, but after 4 hours it was comparable to that of the phosphorylated hexoses (Table IV).<sup>1</sup>

When sedoheptulose-7-phosphate was incubated together with fructose-1,6-diphosphate, the combination was about twice as efficient in forming DHS as was either compound tested singly (Table IV). This finding suggested a series of reactions involving both substrates. One possible product of such reactions might be sedoheptulose-1,7-diphosphate, a recently discovered enzymatic product (16). The high activity of this compound as a substrate will be described in the following paper (10).

### Materials and Methods

Barium G-6-P  $7H_2O$ , barium fructose-6-phosphate, potassium glucose-1-phosphate, magnesium fructose-1,6-diphosphate, and barium ribose-5-

<sup>1</sup> Attempts to demonstrate the utilization of free sedoheptulose by intact cells, either as the sole carbon source or as a stimulant of DHS accumulation, were unsuccessful.



phosphate were obtained from the Schwarz Laboratories, Inc. Barium sedoheptulose-7-phosphate and barium 6-phosphogluconate were generously supplied by Dr B L Horecker. The barium salts were converted to the corresponding potassium salts by adding a slight excess of potassium sulfate and removing the barium sulfate by centrifugation.

Sodium ATP was obtained from the Pabst Laboratories. Hexokinase was kindly supplied by Dr J Gregory. Uniformly  $C^{14}$ -labeled glucose was obtained from the Nuclear Instrument and Chemical Corporation.

Protein concentration was determined spectrophotometrically (17). Radioactive samples were counted under standard conditions at "infinite" thickness on stainless steel dishes of 1 sq. cm. area, and enough counts were taken to give a probable error of 5 per cent.

#### SUMMARY

An extract of a mutant of *Escherichia coli* has been shown to catalyze the chain of reactions from various phosphorylated carbohydrates to 5-dehydroshikimic acid, a precursor of the benzene ring of several aromatic metabolites. This finding makes it possible to test intermediates in this path that cannot be studied with intact cells.

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# THE ENZYMATIC CONVERSION OF SEDOHEPTULOSE-1,7-DIPHOSPHATE TO SHIKIMIC ACID\*

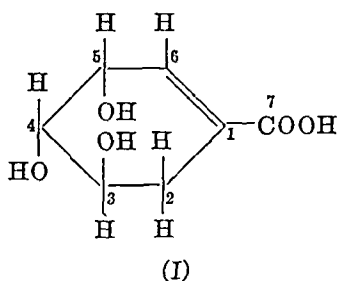
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In the preceding paper (1) extracts of an appropriate mutant of *Escherichia coli* were shown to form 5-dehydroshikimic acid (DHS), though in small yield, from various phosphorylated carbohydrates. It therefore became possible to undertake an enzymatic analysis of the early stages of aromatic biosynthesis.

The present paper will show that such extracts can form DHS (or its immediate metabolic product, shikimic acid (SA)) almost quantitatively from sedoheptulose-1,7-diphosphate (SDP), a compound produced enzymatically from tetrose phosphate plus triose phosphate (2, 3). Furthermore, carbon atoms 4,5,6,7 of SDP were shown by isotopic labeling to be incorporated into SA (I) exclusively in positions 3,4,5,6 and without dilution.



These results, like those on the formation of SA from labeled glucose by intact cells (4), indicate that carbon atoms 3,4,5,6 of SA arise from tetrose

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phosphate and the other 3 carbon atoms from triose phosphate.<sup>1</sup> These studies with labeled glucose, however, show further that formation of SA could not involve cyclization of the intact carbon chain of SDP. A part of the present work has been published in preliminary form (6).

#### EXPERIMENTAL

*Bacterial Extracts, Assay of DHS or SA*—The enzyme system consisted of unfractionated extracts of mutants 83-2 (blocked between DHS and SA) and 83-24 (blocked after SA). The method of preparing the extracts is described in the preceding paper (1). Diphosphopyridine nucleotide (DPN) was removed from certain preparations by treatment with charcoal (1 mg per mg of protein) at 0° for 30 minutes, followed by centrifugation at 0°.

The assay for DHS or SA is described in the preceding paper (1).

*Preparation of SDP-4,5,6,7- $C^{14}$* —SDP labeled in carbon atoms 4,5,6,7 was prepared enzymatically. 64 mg (114  $\mu$ c) of D-ribose uniformly labeled with  $C^{14}$  were converted to sedoheptulose-7-phosphate (7). The material was further converted, in the presence of unlabeled fructose 1,6-diphosphate, to SDP (2), which was isolated as the Ba salt. The over-all yield from ribose was 27 per cent. The BaSDP contained 16.4 per cent sedoheptulose by the orcinol reaction, 4.8 per cent esterified P (potato phosphatase<sup>2</sup>), 6.7 per cent total P, and 1.6 per cent inorganic P. These analyses indicate a purity of 50 per cent and a ratio of P to heptulose of 1.9 (theoretical 2.0).

*Degradation of SDP-4,5,6,7- $C^{14}$* —The distribution of activity in the labeled SDP was established by the following degradation. 21.3 mg of the Ba salt were converted to the potassium salt and dephosphorylated with potato phosphatase<sup>2</sup>. After the addition of an equilibrium mixture (20 per cent heptose) of sedoheptulosan-sedoheptulose (prepared by heating 0.5 gm of sedoheptulosan monohydrate with 50 ml of 1 per cent HCl for 30 minutes over a steam bath (8)) as carrier, sedoheptulose osazone was prepared and was recrystallized from aqueous methanol to constant radioactivity and a reported melting point of 194–197° (8). Yield 130 mg (70 per cent).

The osazone (52 mg) was dissolved (with warming) in 30 ml of 60 per cent ethanol, cooled to 30°, and treated with 1.8 ml of 1 M  $\text{NaHCO}_3$  and 1.8 ml of 0.3 M sodium periodate (9). This procedure yielded the 1,2-bisphenylhydrazone of mesoxalaldehyde (from C-1,2,3), formate (from C-4,5,6), and formaldehyde (from C-7).

<sup>1</sup> Observations on the origin of protocatechuic acid in *Neurospora* are consistent with the same pathway in that organism (5).

<sup>2</sup> Prepared according to an unpublished procedure of Dr. A. Kornberg.

After 1 hour the bisphenylhydrazone was removed by filtration, washed with water, dried *in vacuo*, and recrystallized from 66 per cent ethanol. The combined filtrate and washings were adjusted to pH 6 and treated with 5 ml of 1 M  $\text{SiCl}_2$ . After removal of the precipitated iodate and periodate salts by filtration, the supernatant solution was acidified with acetic acid, freed of  $\text{CO}_2$ , and treated with  $\text{HgCl}_2$  (4, 10). The resulting  $\text{CO}_2$  (from formate representing C-4,5,6) was collected as  $\text{BaCO}_3$ . After removal of  $\text{Hg}_2\text{Cl}_2$  by filtration, the pH of the filtrate was adjusted to pH 4.8 and the formaldehyde (C-7) was isolated as the dimedon derivative (m.p.  $191^\circ$ ).

TABLE I  
Degradation of Sedoheptulose-1,7-diphosphate Labeled  
in Carbon Atoms 4, 5, 6, and 7 with  $\text{C}^{14}$

Compound or atom	Activity*	
	Observed	Calculated†
	<i>c p m</i>	<i>c p m</i>
Ba salt of SDP	265,000‡	
Sedoheptulose osazone	255,000§	
Mesoxalaldehyde 1,2-bisphenylhydrazone (C-1,2,3)	0	0
$\text{BaCO}_3$ (C-4,5,6)	199,000	191,000
Formaldehyde (dimedon derivative) (C-7)	65,000	63,800

\* As in Srinivasan *et al.* (4), counts per minute at "infinite" thickness under standard conditions divided by the fraction of carbon in the compound and multiplied by the number of carbon atoms per molecule. With  $\text{BaCO}_3$  derived from more than 1 carbon atom of SDP the activity was further multiplied by the number of carbon atoms represented.

† From the activity of the osazone on the assumption of uniform labeling in C-4,5,6,7.

‡ Observed activity corrected for 50 per cent purity of the compound (see "Experimental").

§ Corrected for dilution with carrier sedoheptulose.

Recrystallization from methanol gave material with unchanged melting point and activity.

The radioactivities of the various fragments were determined as in the preceding paper (1). The results (Table I) show that the SDP was labeled exclusively in carbon atoms 4, 5, 6, and 7. Furthermore, evidence is provided that these atoms, as expected, are equally labeled.

*Conversion of SDP-4,5,6,7- $\text{C}^{14}$  to SA, Degradation of SA*—In order to obtain a product that could be conveniently degraded, the conversion of the labeled SDP was carried beyond DHS to SA. This additional reaction requires reduced triphosphopyridine nucleotide (TPNH) (11), as well as an appropriate extract (Strain 83-2, used in the previous experiments, is blocked in this reaction).

Pilot experiments were first performed by measuring the conversion of labeled SDP to DHS and SA. Both *Aerobacter aerogenes* strain A170 113-1 which responds to DHS or SA (12), and *E. coli* strain 156 53D2, which responds only to SA (13), were employed in the assays. After incubating SDP for 3 hours with an extract of mutant 83-24 (blocked after SA), the yield of DHS was 75 per cent and that of SA was zero. TPN was then added, along with an excess of isocitrate as a means of regenerating TPN. On further incubation for 10 minutes, the DHS was quantitatively reduced to SA. Furthermore, when the isocitrate and TPN were added at the

TABLE II  
Conversion of Sedoheptulose-1,7-diphosphate to  
5-Dehydroshikimic Acid, DPN Requirement

Experimental conditions	Conversion to DHS	
	1 hr	2 hr
	per cent	per cent
Untreated extract	56	81
“ “ + DPN	56	78
Charcoal-treated extract	0	0
“ “ + DPN	58	75
“ “ + TPN	~5	28

Preparation of the extract of *E. coli* mutant 83-2 and its treatment with charcoal are described under "Experimental." The reaction mixture contained 0.1 ml of extract (20 mg of protein per ml), 0.25  $\mu$ mole of potassium SDP, 50  $\mu$ moles of potassium phosphate buffer (pH 7.4), and 5  $\mu$ moles of  $MgCl_2$  in a total volume of 1 ml. DPN or TPN (0.25  $\mu$ mole) was added as indicated. After 1 and 2 hours at 37°, 0.5 ml of the solution was treated with 0.01 ml of 6 N HCl, the precipitated protein was removed by centrifugation, and the supernatant solution was assayed for DHS (1).

beginning of an incubation with SDP, the yield of SA was again 75 per cent.

In the isotopic experiment, 50  $\mu$ moles of the potassium salt of labeled SDP were incubated with 20 ml of extract of *E. coli* mutant 83-24 (20 mg of protein per ml), 5 mmoles of potassium phosphate buffer, pH 7.4, 0.5 mmole of  $MgCl_2$ , 10 mg of TPN (80 per cent purity), and 4 mmoles of *d*-isocitrate in a total volume of 100 ml. After 3 hours at 37° 1 ml of 6 N HCl was added and the precipitated protein was removed by centrifugation. Aliquots were assayed for SA microbiologically in duplicate at five different concentrations. The yield was  $5.1 \pm 0.2$  mg (29  $\mu$ moles), corresponding to a conversion of 58 per cent. To the remainder of the solution 500 mg of carrier SA were added. SA was then isolated and degraded by previously described procedures (4).

## Results

*Conversion of SDP to DHS*—It was shown in the preceding paper (1) that a mixture of sedoheptulose-7-phosphate and fructose-1,6-diphosphate

TABLE III

*Incorporation of Sedoheptulose Diphosphate-4,5,6,7-C<sup>14</sup> into Shikimic Acid*

Compound or atom	Activity* $\times 10^{-3}$
	<i>c p m</i>
Barium sedoheptulose-1,7-diphosphate	6200†
C 4, 5, 6, or 7 of SDP (calculated)	1550
Shikimic acid	6100‡
Methyl shikimate	6190
<i>trans</i> -Aconitic acid§	4460
Itaconic acid§	3060
C-7,1,2,3 of SA	1550
C 4,5,6	4400
C-1,7	0
C 2,6	1530
C 3,5	2990¶
C 2	0
C-3 (calculated)	1550
C-4	1430
C 5 (calculated)	1440
C 6       “	1530
Carboxyl (C-7)	0

\* As in Table I With BaCO<sub>3</sub> or formaldehyde derived from more than 1 carbon atom of SA the activity was further multiplied by the number of carbon atoms represented (4) In this form the data for the constituent carbons of SA are additive and are comparable to a labeled atom of SDP

† Observed activity corrected for 50 per cent purity of compound (see “Experimental”) In the next line this value is divided by 4 to give activity per labeled atom of SDP

‡ Observed activity corrected for dilution with carrier SA

§ The significance of these compounds and the method of obtaining the activities of the fragments presented have been published (4)

|| We are indebted to Dr. Milon Sprecher for isolating this fragment from SA

¶ A value of 3030 was obtained from CO<sub>2</sub> evolved from oxalacetic acid (4), which also represents C-3,5

was somewhat more efficiently converted to DHS than was either compound tested singly Since these two substrates can interact, in the presence of transaldolase and aldolase, to yield SDP (2), the latter compound was tested by the procedure employed previously (Table II) SDP was found to be much more efficiently converted to DHS than any other sub-

strates tested, the values in various experiments varying from 35 to 80 per cent

*Diphosphopyridine Nucleotide (DPN) Requirement for Conversion*—In synthesis of DHS ( $C_7H_8O_5$ ) from SDP ( $C_7H_{14}O_7$ ) involves the removal of 2 atoms of hydrogen as well as 2 molecules of water. Pyridine nucleotides were therefore investigated as possible cofactors for this reaction sequence. It was found, as shown in Table II, that removal of nucleotides by charcoal treatment of the extract completely eliminated the formation of DHS and subsequent addition of DPN restored full activity. TPN was partially effective. The activity of the untreated extracts presumably depends on the presence of catalytic amounts of DPN, which would be regenerated under the conditions of these experiments by the DPNH oxidase known to be present in *E. coli* extracts (14).

*Incorporation of SDP-4,5,6,7- $C^{14}$  into SA*—In order to obtain further information on the path by which SDP is utilized in this reaction sequence an experiment was performed with SDP-4,5,6,7- $C^{14}$ . Since degradation procedures were available for SA (4) but not for DHS, the conversion was carried beyond DHS to SA, by using an extract of a mutant blocked after SA and providing TPNH, which is required for the additional reaction (11). As shown under "Experimental," the yield was excellent and all the DHS was reduced to SA.

After correction for dilution with carrier SA, the activity of the product was found equal to that of the SDP. The SA was degraded and the results are presented in Table III. It can be seen that carbon atoms 7, 1, and 2 were inactive, while carbon atoms 3, 4, 5, and 6 were each as active as atom 4, 5, 6, or 7 of the labeled SDP.<sup>3</sup> It can be concluded, therefore, that the "bottom" 4 carbon atoms of SDP are incorporated, presumably as a unit, into C-3,4,5,6 of SA, while the "top" 3 atoms give rise to C-7,1,2 of SA.

#### DISCUSSION

With the bacterial extracts used in this investigation SDP, though theoretically subject to a multiplicity of reactions, is largely converted to DHS (or SA). Furthermore, in this conversion, carbon atoms 1 to 3 of SDP give rise to carbon atoms 7,1,2 of SA, while carbon atoms 4 to 7 of SDP give rise to carbon atoms 3 to 6 of SA.

These results could be explained by cyclization of the intact 7-carbon

<sup>3</sup> It is clear (cf. "Discussion") that these results would be expected theoretically. This sample of SA can therefore provide a direct test of the degradation procedure. It may be observed from the results in Table III that the activities of the various fragments, determined in different ways (4), are in excellent agreement with each other and with the expected values (cf. (4), footnote 2). Even the direct determination of C-4,5,6, which had given trouble earlier (cf. (4), footnote 3), now proves reliable.

chain of SDP during its conversion to SA. However, this hypothesis is eliminated by the results of other studies, as illustrated in Fig 1. Thus in the only known reaction for forming SDP, *ie* condensation of triose phosphate with tetrose phosphate under the influence of aldolase (3), carbon atoms 1, 2, and 3 of SDP would be derived from atoms (1,6), (2,5), and (3,4) of glucose, respectively. Cyclization of the intact chain of SDP would then yield SA with this sequence in positions C-7, C-1, and C-2, respectively. However, the *reverse* sequence of glucose atoms was actually found in these positions in SA formed from specifically labeled glucose by intact cells (4). It therefore appears that in the present experiments fragment 1,2,3 of SDP was detached and "inverted" prior to incorporation into SA.

This interpretation has been supported by the results of further work

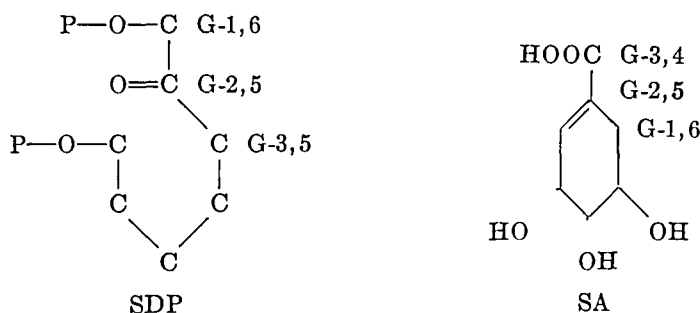


FIG 1 Incorporation of carbon atoms of glucose (G-1, G-2, ) into SDP (2, 3) and SA (4)

with these bacterial extracts, which showed that the formation of SA from SDP involves cleavage to D-erythrose-4-phosphate plus triose phosphate and conversion of the triose phosphate to phosphoenolpyruvate (15). Since the latter reaction sequence is known to involve DPN, an explanation was provided for the observation (Table II) that DPN is required for the conversion of SDP to SA. Furthermore, since triose phosphate and tetrose phosphate can be derived from glucose by pathways not involving SDP, the latter compound, despite its excellent utilization, does not appear to be an obligatory intermediate in aromatic biosynthesis.

### Materials and Methods

We are grateful to Dr B L Horecker for barium sedoheptulose-7-phosphate and barium sedoheptulose-1,7-diphosphate, to Dr N K Richtmyer for sedoheptulosan monohydrate, and to Dr S Ochoa for the dimethyl ester of the lactone of *D*-isocitric acid (obtained from Dr H B Vickery).

Magnesium fructose-1,6-diphosphate and uniformly  $C^{14}$ -labeled ribose



were purchased from the Schwarz Laboratories, Inc. TPN and DPA were obtained from the Pabst Laboratories.

The methods used for determining protein concentration and radioactivity were described in the preceding paper (1).

It is a pleasure to acknowledge our many stimulating discussions with Dr. B. L. Horecker and the generous hospitality he accorded for the enzymatic synthesis of labeled sedoheptulose-1,7-diphosphate.

#### SUMMARY

Sedoheptulose-1,7-diphosphate (SDP) was shown to be converted almost quantitatively to shikimic acid (SA) by extracts of a mutant of *Escherichia coli* blocked after SA. Diphosphopyridine nucleotide is required in this process. Furthermore, when the conversion was carried out with SDP labeled equally in C-4,5,6,7 with  $C^{14}$ , the label was incorporated exclusively and without dilution into C-3,4,5,6 of the ring of SA. The findings are discussed in relation to the results of studies on the biosynthesis of SA from labeled glucose.

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# ACTIVITY OF RESPIRATORY ENZYMES AND ADENOSINE-TRIPHOSPHATASE IN FRAGMENTS OF MITOCHONDRIA\*

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It is now well established that the electron carriers which constitute the respiratory chain between pyridine nucleotide and cytochrome oxidase are largely localized in the mitochondria. Although organized activity of the citric acid cycle and the fatty acid oxidation cycle is lost when mitochondria are disrupted by certain chemical or mechanical procedures, the particulate fragments so formed still retain the capacity to carry out enzymatic reactions involved in terminal electron transport. For instance, the widely studied respiratory granules of Keilin and Hartree (1, 2) which contain the cytochrome system and can oxidize succinate or DPNH have been shown by Cleland and Slater (3) to be fragments of heart muscle mitochondria. More recently, particulate preparations which show considerable activity in terminal electron transport have been obtained by fragmentation of mitochondria isolated by the sucrose method. Fragmentation has been carried out by chemical procedures (4, 5) and also by essentially mechanical methods such as sonic vibration or application of shearing forces (6, 7). The resulting particulate preparations have been suggested to arise from the membranes or cristae of the mitochondria (8, 9). In the cases mentioned these terminal oxidase preparations show no ability to couple phosphorylation to respiration (3, 10), it is presumed that the labile ancillary enzymes and cofactors required for oxidative phosphorylation have been lost in the fragmentation and isolation process.

In work recently reported from this laboratory (11), fragments of relatively low particle weight have been prepared from rat liver mitochondria by the use of digitonin as a dispersing agent. These preparations contain the respiratory carriers required for oxidation of  $\beta$ -hydroxybutyrate or

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<sup>1</sup> Abbreviations BOH,  $\beta$ -hydroxybutyrate, ATP and ADP for adenosine tri- and diphosphates, respectively, CTP, CDP, GTP, GDP, ITP, IDP, UTP, and UDP, for the corresponding tri- and diphosphates of cytidine, guanosine, inosine, and uridine, DPN<sup>+</sup> and DPNH for oxidized and reduced diphosphopyridine nucleotide, respectively, DNP, 2,4-dinitrophenol, P<sub>i</sub>, inorganic phosphate, CoA, coenzyme A

succinate by molecular oxygen via the cytochrome system. However, they differ strikingly from the preparations described above in that they also catalyze coupled phosphorylations with high efficiency. The nature of the differences in enzymatic constitution between phosphorylating and non-phosphorylating fragments of mitochondria therefore is a matter of importance in efforts to reconstruct oxidative phosphorylation in submitochondria.

This report deals with the terminal respiratory activity of particulate fragments of mitochondria obtained by a mild mechanical procedure and also presents a comparison of various enzymatic properties of such non-phosphorylating mitochondrial fragments with those of the phosphorylating digitonin-dispersed fragments. It was found that fragmentation of rat liver mitochondria yields particles with a relatively constant content of certain respiratory enzyme systems regardless of the method of fragmentation employed, whether chemical or mechanical. This characteristic respiratory enzyme distribution was also essentially independent of particle size. The most striking and significant differences found between phosphorylating and non-phosphorylating mitochondrial fragments involve the ATPase activity and the content of bound DPN.

#### EXPERIMENTAL

*Preparation of Mitochondria*—Mitochondria were separated from 0.25 M sucrose homogenates (12) of rat liver (males of Wistar strain, average weight 200 gm). They were washed three times with 0.25 M sucrose, the fluffy layer was removed each time by careful decantation.

*Mechanical Fragmentation of Mitochondria*—This was accomplished by subjecting them to rapid vibration in a partial vacuum in the presence of small glass beads.<sup>2</sup> After the third washing the mitochondrial pellet, about 1.0 to 2.0 ml in volume, was pipetted directly into the lower chamber of a drawn out test tube (15 mm, inside diameter). The chamber was filled with 2 gm of Ballottini glass beads (0.6 mm in diameter) which had been added to the dry tube previously. The tube was placed in an ice bath and evacuated with a pump until active boiling of the mitochondrial suspension occurred. The lower chamber (total volume = 4 ml) was then sealed off with a flame, and was fixed on the reed of a Mickle tissue disintegrator (C. A. Brinkmann and Company) in a cold room. Vibration was carried out with a 10 mm excursion at 50 cycles per second for 10 minutes. The temperature rise was no more than 5°. The material was then subjected to centrifugal fractionation as indicated in Tables I to VII.

*Chemical Fragmentation of Mitochondria*—Two methods were employed. In the first, the washed mitochondrial pellet derived from 10 gm of whole

<sup>2</sup> The use of the partial vacuum was suggested by M. E. Pullman and E. Kinsman.

liver was suspended in 10 ml of cold 1 per cent digitonin solution (Fisher, recrystallized) for 20 minutes (see Cooper and Lehninger (11)). The sedimented fractions collected were washed with cold  $H_2O$  to remove excess digitonin prior to enzymatic assay.

A second method of chemical fragmentation employed 1 per cent sodium cholate as dispersing agent. It was used in exactly the same manner as described above for the case of digitonin. The sedimented fractions were washed with  $H_2O$  to remove excess cholate prior to assay.

*Enzyme Assays*—The multienzyme systems responsible for oxidation of succinate, D- $\beta$ -hydroxybutyrate, and ascorbate by molecular oxygen are designated *succinoxidase*,  *$\beta$ -hydroxybutyric oxidase*, and *cytochrome oxidase*, respectively. The succinoxidase system presumably includes succinic dehydrogenase and cytochromes *b*, *c*, *a*, and *a<sub>3</sub>*, and was assayed manometrically in a buffered system containing succinate. The  *$\beta$ -hydroxybutyric oxidase* presumably includes  *$\beta$ -hydroxybutyric dehydrogenase*, DPN, cytochrome *c* reductase, cytochromes *c*, *a*, *a<sub>3</sub>*, and, in phosphorylating systems, probably cytochrome *b* as well as other factors. It was assayed in the presence of added substrate, DPN, and buffer. Cytochrome oxidase was measured with ascorbic acid as reductant of cytochrome *c*. The details of the assays are given in Tables I to VII, the reported activities being based on the maximal initial rates observed.

The rate of reduction of ferricytochrome *c* by various substrates was measured spectrophotometrically at 550  $m\mu$ . Initial rates are recorded.

ATPase activity was assayed by measuring liberation of  $P_i$  from ATP in the presence or absence of added  $Mg^{++}$  or 2,4-dinitrophenol, as shown. When fresh mitochondria were assayed for ATPase, they were first preincubated at 37° for 10 minutes to evoke the latent ATPase (13–15).

It is emphasized that the measurements of activity of both the respiratory enzyme systems and probably also the ATPase activity are essentially those of rate-limiting steps in complex multienzyme sequences.

### Results

*Oxidative and ATPase Activities after Mechanical Fragmentation*—Typical data in Table I show the effect of mechanical fragmentation on four mitochondrial enzyme systems. It is seen that approximately 50 to 75 per cent of the “oxidase” activities of intact mitochondria could be recovered after the disruption, *ie* as Fraction II (see Table I). Most of this surviving activity in turn was in a form not sedimentable at  $25,000 \times g$  but sedimented at  $100,000 \times g$ , *ie* Fraction IV. The  $25,000$  to  $100,000 \times g$  fraction thus accounts for about 50 per cent of the original oxidase activity and about one-third of the original total N of the mitochondria.

Assay of the ATPase activity indicated an increase after fragmentation,

suggesting that some additional latent activity appeared upon fragmentation, possibly by increasing accessibility of the substrate to the enzyme. As in the case of the respiratory systems, most of the ATPase was found in the fraction sedimented at 25,000 to 100,000  $\times g$ . It may be noted that the ATPase activity of this fraction is high and, on a molar basis, substantially greater than the absolute activity of the succinoxidase or BOH-oxidase systems, such high activity is compatible with but does not

TABLE I

*Yields of Enzymatic Activities after Mechanical Fragmentation of Mitochondria*

Oxygen uptakes were measured manometrically with a system containing substrates at a concentration of 0.01 M in phosphate buffer 0.02 M, pH 7.4, in a total volume of 1 ml at 25°. The vessels containing BOH and ascorbate were supplied with DPN (0.003 M) and cytochrome *c* (0.00005 M), respectively. The specific activity is expressed as microatoms of oxygen uptake per hour per mg of nitrogen. ATPase was assayed by measuring  $P_i$  liberation by a system with ATP at a concentration of 0.01 M and  $MgCl_2$  at 0.002 M in histidine buffer (0.03 M, pH 7.4), in a total volume of 0.2 ml at 25°. The specific activity is expressed as  $\mu$ moles of  $P_i$  liberation per hour per mg of nitrogen.

Fraction No		N recovery	Succinoxidase		$\beta$ -Hydroxy-butyric oxidase		Cytochrome oxidase		ATPase	
		Per cent total	Per cent total	Specific activity	Per cent total	Specific activity	Per cent total	Specific activity	Per cent total	Specific activity
I	Mitochondria	100	100	28.2	100	34.8	100	232	100	85
II	Fragmented mitochondria	100	45	12.6	77	26.4	57	132	143	118
III	0-25,000 $\times g$	14								
IV	25,000-100,000 $\times g$	32	55	48.0	56	56.0	47	340	92	233
V	100,000 $\times g$ supernatant	54								

\* Mitochondria aged prior to assay by incubating at 37° for 10 minutes in 0.2 M sucrose.

prove that the ATPase activity may be a reflection of the activity of a portion of the enzymatic mechanism which couples phosphorylation to oxidation (15-17). The  $Mg^{++}$  requirement of the ATPase is considered below.

*Relative Activities As Function of Particle Size*—Upon mechanical fragmentation of mitochondria, four fractions were collected by differential centrifugation, Fraction I (10,000 to 25,000  $\times g$ ), Fraction II (25,000 to 50,000  $\times g$ ), Fraction III (50,000 to 100,000  $\times g$ ), and Fraction IV, the supernatant fluid obtained after centrifugation at 100,000  $\times g$ . A spectrum of particles was thus obtained which presumably differed in mass. It is seen (Table II) that the three particulate fractions show approximately

the same specific activities, per mg of total N, of succinoxidase, BOH-oxidase, cytochrome oxidase, and  $Mg^{++}$ -stimulated ATPase. These findings demonstrate that fragmentation of the mitochondrial structure yields a spectrum of particles, all of which have relatively constant enzymatic constitution with respect to the respiratory and ATPase activities studied. They also indicate that no "fractionation" of activities occurs during this form of mechanical disintegration and suggest that all parts of the mitochondrial structure which contribute to the particulate fractions, presumably membranes and cristae (8, 9), contain similar respiratory units made up of the electron carrier chain and the ATPase activity, which possibly

TABLE II

*Enzymatic Activities in Centrifugal Fractions of Fragmented Mitochondria*

The measurements were made in histidine buffer (0.01 M) at pH 6.5, otherwise, the experimental conditions and units of specific activity are those described in Table I.

Centrifugal Fraction No		N recovery	Succin oxidase	$\beta$ -Hydroxy butyric acid oxidase	Cytochrome oxidase	ATPase
		Per cent of total	Specific activity			
I	10,000-25,000 $\times g$	10.7	20.0	31.2	142	122
II	25,000-50,000 $\times g$	17.4	24.0	53.0	158	165
III	50,000-100,000 $\times g$	6.4	27.2	63.6	189	172
IV	Supernatant fraction after 100,000 $\times g$	52.1	2.0	3.8	13	15

represents part of the enzymatic equipment for coupling phosphorylation to the oxidation.

The presence of the oxidase systems and ATPase activity in the supernatant fluid which remains after centrifugation at 100,000  $\times g$  in the same characteristic ratios as in the particulate fractions suggests that very small particles, representing the smaller end of the spectrum of particle size, are present in this fraction and are quite similar in enzymatic constitution to the larger particles. This fraction also contains considerable soluble protein. None of the particulate fractions obtained by the mechanical fragmentation described was found capable of coupling phosphorylation of ADP to oxidation or of catalyzing the ATP-phosphate exchange reaction (18), the tests were made by a very sensitive isotopic method (19).

*Comparison of Enzymatic Activities after Mechanical and Chemical Fragmentation*—Since mitochondrial structure may be disrupted by chemical agents, such as digitonin or sodium cholate, to produce respiratory particles

which can be sedimented in high centrifugal fields, it became of interest to examine the oxidase and ATPase activities of such preparations. The choice of these chemical agents was made because one of them, digitonin yields mitochondrial fragments capable of oxidative phosphorylation whereas sodium cholate yields respiratory particles without significant phosphorylating activity.

The data in Table III demonstrate strikingly that the three quite different fragmentation methods used all produce respiratory particles which sediment at 25,000 to 75,000  $\times g$  and have essentially similar oxidase and ATPase activities per mg of total N. The constancy of the specific activities, and hence the constancy of the activities relative to each other despite the use of three widely different fragmentation procedures, provide

TABLE III  
*Comparison of Oxidase and ATPase Activities of Particles  
Obtained by Mechanical and Chemical Fragmentation*

The assay conditions were those described in Table I. Specific activities are based on nitrogen content, see Table I.

Type of particle	Succinoxidase	$\beta$ -Hydroxy butyric acid oxidase	Cytochrome oxidase	ATPase
	Specific activity			
Mechanical	32	51	164	180
Digitonin*	30	32	146	133
Cholate	35	41	123	173

\* Data recalculated from Cooper and Lehninger (11, 21)

still more evidence for the existence of characteristic and relatively durable respiratory enzyme units which are not readily dissociated, at least by the procedures used.

*Dehydrogenase Content of Mitochondrial Fragments*—All particulate fragments obtained from rat liver mitochondria by the methods described have been found to contain both D- $\beta$ -hydroxybutyric dehydrogenase (DPN-linked) and succinic dehydrogenase. Although succinic dehydrogenase has been obtained in soluble form from animal tissue mitochondria (5), a soluble preparation of D- $\beta$ -hydroxybutyric dehydrogenase has never been described. Work in this laboratory has indicated that it is more tightly bound to the respiratory particles than succinic dehydrogenase.\*

Examination of these particulate fragments for other dehydrogenases has revealed that preparations made by the mechanical, digitonin, and

\* Sudduth, H. C., unpublished observations.

cholate methods also contain some L-malic dehydrogenase (DPN-linked), choline dehydrogenase, and glutamic dehydrogenase. Only in the case of malic dehydrogenase do the measurements suggest that it may, like succinoxidase and  $\beta$ -hydroxybutyric dehydrogenase, be a constant component enzyme of the respiratory particles described. However, as will be pointed out, the malic dehydrogenase, although DPN-linked, does not appear to react with the respiratory chain in the same manner as the D- $\beta$ -hydroxybutyric dehydrogenase. The content of choline and glutamic dehydrogenases is low and variable. The particles contain little or no fumarase,

TABLE IV  
*Presence of Functional Bound DPN*

Reduction of cytochrome *c* was measured spectrophotometrically at 550 m $\mu$  at 24° in the following test system, 0.003 M BOH, 0.01 M phosphate, pH 7.4,  $5 \times 10^{-5}$  M ferri-cytochrome *c*, 0.003 M KCN, and enzyme preparation in a total volume of 3.0 ml. Antimycin A was added at a level of 1.0  $\gamma$  per ml. The specific activity is expressed as micromoles  $\times$  2 cytochrome *c* reduced per hour per mg. of N.

Type of preparation	DPN added	Specific activity	
		Without antimycin	With antimycin
	M		
Mechanical	None	0.4	<0.2
Digitonin	"	11.4	<0.2
Cholate	"	<0.2	<0.2
Mechanical	0.0002	78	74
Digitonin	0.0002	19.5	11.7
Cholate	0.0002	50	43

and  $\alpha$ -ketoglutarate is not oxidized at a significant rate when tested with supplements of CoA and GDP.

*"Bound" DPN*—In searching for differences between the properties of mitochondrial particles prepared with digitonin and those prepared by mechanical disintegration, in order to account for the fact that the former couple phosphorylation to oxidation whereas the latter do not, it was found that the digitonin particles contain bound DPN functional with bound D- $\beta$ -hydroxybutyric dehydrogenase, whereas the particles prepared mechanically or by means of cholate do not. Typical data summarized in Table IV demonstrate that reduction of cytochrome *c* by D- $\beta$ -hydroxybutyrate in the digitonin preparation occurs in the absence of added DPN, in confirmation of earlier work in this laboratory (20). However, only a trace of reduction could be observed in the absence of added DPN under similar conditions with the "mechanical" and "cholate" preparations. The reduc-



tion of cytochrome *c* by D- $\beta$ -hydroxybutyrate which occurs via bound DPN was completely inhibited by antimycin A

When free DPN was now added to this reaction medium, then the cholate and mechanical preparations showed high activity in reduction of cytochrome *c* by BOH, however, this was not inhibited by antimycin A. Addition of DPN to the digitonin particle system caused only a limited increase in the rate of reduction of cytochrome *c*, but this increment was not inhibited by antimycin A. It has been demonstrated by Devlin and Lehninger (20) that only the antimycin-sensitive reduction of cytochrome *c* via the bound DPN present in the digitonin particle is accompanied by coupled phosphorylation of ADP. In similar experiments the particles obtained by the mechanical procedure or by the use of cholate were found to be

TABLE V  
*Availability of Bound DPN to Bound Dehydrogenases*

Test system as in Table IV The enzyme was digitonin preparation

Substrate	DPN added	Reduction of cytochrome <i>c</i>
		Specific activity
D- $\beta$ -Hydroxybutyrate	M	11.1
L-Malate	None	<0.2
D- $\beta$ -Hydroxybutyrate	0.0002	19.0
L-Malate	0.0002	52

completely incapable of coupling phosphorylation to reduction of cytochrome *c* by D- $\beta$ -hydroxybutyrate in the presence of added DPN.

The bound DPN in the digitonin particle, although functional with D- $\beta$ -hydroxybutyric dehydrogenase, is not functional with the L-malic dehydrogenase present in these particles (Table V), addition of free DPN causes a high rate of antimycin-insensitive reduction of cytochrome *c* which in other experiments has been found to be unaccompanied by phosphorylation. From these findings it appears probable that not only the presence of bound DPN in the mitochondrial fragment but also its ability to react with the bound dehydrogenase in the fragment may be a prerequisite for the occurrence of coupled phosphorylation at the pyridine nucleotide level of the respiratory chain.

*Nucleoside Tri- and Diphosphatase Activity*—Particulate preparations from mechanically fragmented mitochondria have considerable ATPase activity in the presence of added  $Mg^{++}$ , as already shown (7). The data summarized in Table VI confirm this finding and demonstrate that in the

absence of added  $Mg^{++}$  there is only feeble activity, which is not stimulated significantly by DNP. Likewise, there is little activity against other nucleoside 5'-triphosphates in the absence of  $Mg^{++}$ . However, when  $Mg^{++}$  is present, there is a high rate of hydrolysis of ATP which is doubled by addition of DNP. In addition, ITP and GTP are rapidly hydrolyzed, whereas CTP and UTP are hydrolyzed relatively slowly.

TABLE VI

*Nucleoside 5'-Triphosphatase of Mitochondrial Fragments*

The conditions of the assay are described in Table I. The mechanical fragments were those sedimenting between 25,000 and 100,000  $\times g$ . The data on the fragments obtained by treatment of mitochondria with digitonin are recalculated, on the same activity basis, from the data of Cooper and Lehninger (21) Table I.

Substrate	MgCl added	Specific activity, $\mu$ moles of $P_i$ per hr per mg N	
		Mechanical	Digitonin
ATP	$\mu$ None	4.8	18.6
" + DNP	"	6.1	79.0
CTP	"	3.2	1.2
GTP	"	2.8	3.6
ITP	"	4.6	2.4
UTP	"	2.6	1.8
ATP	0.002	111.0	133.0
" + DNP	0.002	228.0	132.0
CTP	0.002	9.3	6.0
GTP	0.002	52.0	45.0
ITP	0.002	101.0	80.4
UTP	0.002	16.2	15.6

Since the ATPase activity of mitochondria has been associated with the mechanism of the coupling of phosphorylation to oxidation (15-17), a detailed comparison was made of the nucleoside 5'-triphosphatase of the non-phosphorylating fragments prepared mechanically with the phosphorylating fragments prepared with digitonin. Table VI also contains data for the digitonin particles (21), recalculated to the same activity basis. From this comparison it is seen that the specific rate of hydrolysis of CTP, GTP, ITP, and UTP in the presence or absence of added  $Mg^{++}$  is almost identical in the two types of mitochondrial fragments. On the other hand, the hydrolysis of ATP differs strikingly in the two preparations. In the absence of added  $Mg^{++}$  the mechanical preparation has little ATPase activity which is not stimulated significantly by DNP. However, the digitonin

particles have much higher ATPase activity in the absence of  $Mg^{++}$ , which, in contrast, is greatly stimulated by DNP.

Another point of difference is revealed when 0.002 M  $Mg^{++}$  is present. Under these circumstances both the mechanical and digitonin preparations hydrolyze ATP at about the same rate. However, when DNP is also added, the mechanical preparation shows a large stimulation, whereas the digitonin preparation shows none. In these assays ample ATP was present, a limited supply of substrate in the test system was not the cause of the failure to observe stimulation by DNP.

Various nucleoside 5'-diphosphates were tested in a similar manner (Table VII), but showed low rates of hydrolysis even in the presence of  $Mg^{++}$ . The nucleoside 5'-diphosphatase of mitochondria studied by Plaut

TABLE VII  
*Nucleoside 5'-Diphosphatase Activity*

Assays as in Table I, all were carried out in the presence of 0.002 M  $MgCl_2$ . The supernatant fraction (see the text) was dialyzed 20 hours against 0.005 M in 0.1 M (2,2,2-trimethyl)aminomethane buffer, pH 7.5, at 0° prior to assay.

Substrate	Specific activity, $\mu$ moles $P_i$ per hr per mg N	
	Mechanical particles	Supernatant fraction
ADP	0.90	2.0
CDP	0.24	2.6
GDP	0.70	21.7
IDP	0.06	10.7
UDP	0.14	15.5

(22) and Gregory (23) was found not to be present in the particulate fractions. However, the dialyzed supernatant fluid which remained after sedimentation of mechanically fragmented mitochondria at 100,000  $\times g$  was found to contain what appeared to be this diphosphatase activity (see Table VII), since, in agreement with the findings of Plaut, GDP, IDP, and UDP were readily hydrolyzed, whereas ADP was not.

#### DISCUSSION

The results of this study of the distribution of certain enzyme systems among particulate fragments of mitochondria disintegrated by a variety of procedures suggest that the individual enzymes which make up the respiratory chain are present in the original insoluble structures of the mitochondria, presumably the membranes and cristae (8, 9), in a rather fixed and definite proportion. Furthermore, they suggest that all portions of the membranes and cristae which contribute to the insoluble particulate fraction

tions contain about the same amount of respiratory activity per mg of nitrogen. It is therefore quite possible that highly organized multienzyme respiratory units are more or less uniformly distributed in all parts of the membranes and cristae, since the smallest particulate fragments show the same characteristic enzyme pattern and activity per mg of N as the largest fragments. It is in fact conceivable that the membranes and cristae are composed of relatively durable respiratory complexes or units, imbedded in a regular manner in a structural fabric in which the individual respiratory units are separated from each other by relatively fragile points of attachment which are easily broken by low frequency vibrations or by such agents as cholate or digitonin.

Although the enzymatic composition of the fragments was found to be independent of the mode of fragmentation and, in the case of mechanically fragmented mitochondria, independent of particle size, evidently more drastic methods of treatment are capable of removing one or another enzyme from the unit. In this way succinic dehydrogenase has recently been obtained as a soluble protein in apparently pure form by Kearney and Singer (24). On the other hand, other enzymes such as cytochromes  $a$ ,  $a_3$ , and D- $\beta$ -hydroxybutyric dehydrogenase are not so easily removed from the mitochondrial membrane.

The differences observed in the content of bound and functional DPN between the phosphorylating digitonin fragments and the non-phosphorylating mechanical fragments are considered highly significant. Earlier work has shown that the phosphorylating digitonin preparations can readily oxidize added DPNH but no phosphorylation was found to be coupled to this oxidation, at least in the span between DPN and cytochrome  $c$  (20). It appears possible from these findings that the apparent requirement for bound DPN in coupling phosphorylation at the pyridine level of the respiratory chain may be a reflection of the *in situ* generation of a chemical species or a derivative of DPNH which is formed uniquely only when the  $\text{DPN}^+$  is enzymatically reduced in the bound form, according to a general reaction mechanism postulated by one of us earlier (25). Enzymatic reduction of free  $\text{DPN}^+$  would yield free DPNH, which itself is not accessible to the phosphorylating pathway (20).

The ATPase activities of the two types of mitochondrial fragments are also significantly different. The non-phosphorylating fragments show virtually no ATPase activity in the absence of added  $\text{Mg}^{++}$  even with DNP, but in the presence of  $\text{Mg}^{++}$  they have high activity, which in turn is greatly stimulated by DNP. On the other hand, the phosphorylating digitonin fragments have significant ATPase in the absence of added  $\text{Mg}^{++}$  which is greatly stimulated by DNP. Addition of  $\text{Mg}^{++}$  evokes additional activity which cannot be stimulated further by DNP. The digitonin frag-

ments catalyze the incorporation of labeled orthophosphate into ATP, in the absence of electron transport (26), the mechanical fragments are completely devoid of this activity. The meaning of these differences is not clear at present, however, it appears probable that mitochondrial ATPase activity is not due to the action of a single purely hydrolytic enzyme but possibly to the action of a more complex enzyme system concerned with oxidative phosphorylation. One step of this system may be susceptible to inactivation, especially after mechanical fragmentation of mitochondria which causes a purely hydrolytic activity to appear which normally does not participate in phosphate transfer. It is also possible that two separate ATPase activities are present, one of which may be irrelevant to coupling of phosphorylation. The nature of the enzymatic difference between phosphorylating and non-phosphorylating fragments is being investigated further.

#### SUMMARY

When rat liver mitochondria are fragmented by mechanical vibration, a spectrum of particles differing in rate of sedimentation is formed. About 50 per cent of the original succinoxidase,  $\beta$ -hydroxybutyric oxidase, cytochrome oxidase, and ATPase activities are recovered in the particulate fractions. Over a wide range of particle size, the absolute activity of each of these systems and, hence, the ratio of activities are approximately constant. Similarly, chemical fragmentation of rat liver mitochondria by treatment with digitonin or sodium cholate solutions yields particles of about the same sedimentation characteristics. The absolute activities of these enzyme systems and their ratios in the chemically fragmented mitochondrial particles are almost identical with those of fragments produced mechanically. These findings suggest that the mitochondrial membrane and cristae may contain, possibly in a uniformly distributed manner, relatively durable respiratory units of constant (enzymatic) composition separated by relatively fragile points of attachment.

The digitonin fragments, which couple phosphorylation to oxidation, contain bound DPN, which is functional with bound D- $\beta$ -hydroxybutyrate dehydrogenase but not with bound malic dehydrogenase, and also have DNP-stimulated ATPase activity in the absence of added  $Mg^{++}$ . The cholate or mechanical fragments, which do not couple phosphorylation to oxidation, lack these properties. These differences are considered to be significant in the enzymatic organization of phosphorylating *versus* non-phosphorylating fragments of mitochondria.

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# ACTION OF PROTEOLYTIC ENZYMES ON SOME PEPTIDES AND DERIVATIVES CONTAINING HISTIDINE\*

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Despite the importance of histidine as a constituent of proteins, there is, as yet, little information concerning the susceptibility of peptides or peptide derivatives containing this amino acid to the action of proteolytic enzymes, largely because only a few such compounds have been available. An earlier investigation from this laboratory described the partial purification and properties of carnosinase (1), an enzyme from swine kidney which hydrolyzes carnosine and certain other histidine-containing peptides. The present study describes the synthesis of a number of peptides and derivatives of L-histidine and their susceptibility to several proteolytic enzymes.

Bergmann *et al* (2) showed that L-lysyl-L-histidine was hydrolyzed rapidly by the "dipeptidase" of swine intestinal mucosa, but that it was not hydrolyzed by trypsin or "aminopolypeptidase." Trypsin also failed to hydrolyze benzoyl-L-histidinamide (3). Bergmann and Fruton (4) demonstrated that papain hydrolyzes benzoylglycyl-L-histidinamide between the glycine and histidine residues at about one-twelfth the rate of  $\alpha$ -benzoyl-L-argininamide. Reports that certain peptides of histidine can serve as a source of this amino acid for growth in rats (5) and in *Lactobacillus delbrueckii* (6) also indicate the existence of enzyme capable of hydrolyzing such peptides.

Little is known about the enzymatic hydrolysis of peptides containing the carboxyl group of histidine in peptide linkage, since a satisfactory method for their synthesis became available only recently. Indirect evidence, however, has come from amino acid sequence studies with proteolytic enzymes. Locker (7) reported that carboxypeptidase releases histidine from actin. Hill and Smith (8) have shown that highly purified leucine aminopeptidase releases histidine from the oxidized B chain of insulin, in agreement with earlier results of Smith and Spackman (9) which indicated that the aminopeptidase hydrolyzes histidinamide and other compounds.

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In order to provide a larger variety of histidine substrates, a method was developed for incorporating the carboxyl group of histidine in peptide linkage, an achievement which had not been reported when this study was initiated. While this work was in progress, Holley and Sondheimer (10) published an almost identical procedure, and, shortly thereafter, Fischer and Whetstone (11) also reported a similar method.

### *Enzymatic Studies*

*Methods*—All the enzymatic experiments were performed at 39° in 25 ml volumetric flasks. The test solutions contained 0.05 M substrate. The extent of hydrolysis was determined by the microtitration method of Grassmann and Heyde (12). The results are expressed as the proteolytic coefficient,  $C_1$  or  $C_0$ , where  $C_1 = k_1/E$  and  $C_0 = k_0/E$ , or as the rate relative to that of a known typical substrate as 100.  $E$  is the enzyme concentration in mg of protein N per ml and was determined by the procedure of Bucher (13).  $k_{1t} = \log (100/100 - \chi)$ , where  $\chi$  is the percentage hydrolyzed at time  $t$  and correspondingly  $k_{0t} = \chi$ .

*Leucine Aminopeptidase*—Highly purified enzyme was prepared by the method of Spackman, Smith, and Brown (14). Hydrolysis was measured at pH 8.8 or 9.0 in the presence of 0.04 M Tris (tris(hydroxymethyl)amino methane) buffer with both the  $Mn^{++}$ - and  $Mg^{++}$ -activated preparations under the conditions previously described (9). The results shown in Table I indicate that this enzyme readily hydrolyzes L-histidinamide and peptides containing the carboxyl group of histidine in peptide linkage. The failure to hydrolyze carnosine ( $\beta$ -alanyl-L-histidine) is due undoubtedly to the presence of the  $\beta$ -amino group, since glycyl-L-histidine is slowly hydrolyzed by leucine aminopeptidase.

*Carboxypeptidase*—A sample of beef pancreatic carboxypeptidase, four times recrystallized (15), was used. For the less soluble substrates, assays were performed in 35 per cent methanol (16). The results are shown in Table II. For the action of carboxypeptidase on carbobenzoxydipeptides, it is known that the nature of the residue bearing the free carboxyl group has a profound influence on the sensitivity of the substrate, whereas variations in the adjacent amino acid have a much smaller effect (17). The most sensitive substrates possess an aromatic side chain on the carboxyl terminal residue. It is, perhaps, somewhat surprising that at pH 7.5 carbobenzoxyglycyl-L-histidine is such a poor substrate, inasmuch as the imidazole ring is relatively uncharged and might be expected to possess an aromatic character. Apparently this is not the case, since this substrate is hydrolyzed at about 0.005 times the rate of carbobenzoxyglycyl-L-phenylalanine. This finding may have some importance with respect to

TABLE I

*Hydrolysis of L-Histidine Derivatives by Leucine Aminopeptidase\**

Highly purified leucine aminopeptidase was used at the concentrations indicated. Enzyme activation was achieved with 0.001 M  $Mn^{++}$  or 0.004 M  $Mg^{++}$  by incubation for 15 minutes at 40°. Assays were performed at 39° with 0.05 M substrate and 0.04 M Tris buffer at pH 8.8 when  $Mn^{++}$  was the activator and at pH 9.0 with  $Mg^{++}$ .

Substrate	$Mn^{++}$ activation			$Mg^{++}$ activation		
	Enzyme concentration	$C_0$	Relative rate	Enzyme concentration	$C_0$	Relative rate
	$\gamma$ protein N per ml			$\gamma$ protein N per ml		
L-Leucinamide	0.082	14,000	100	0.163	6600	100
L-Histidinamide	0.541	2,700	19	0.543	680	10
L-Histidylglycine	0.137	5,600	40	0.163	580	9
$\beta$ -Alanyl-L-histidine	1.64	0	0			
L-Histidylglycylglycine†	0.274	5,800	41			
Glycyl-L-histidine	3.68	420	3			

\* Some of these results have been given elsewhere (9)

† Analytical figures for this compound are not given in Table VI since these indicated that the compound was not completely pure, however, since it was hydrolyzed by the enzyme preparation, the values are given for comparison

TABLE II

*Action of Carboxypeptidase on Substrates Containing Histidine*

Crystalline carboxypeptidase in 1 M NaCl was used at the concentrations indicated. Assays were performed at 39° with 0.05 M substrate and 0.04 M Tris buffer, pH 7.5. For the less soluble substrates assays were made in 35 per cent methanol, other conditions being the same.

Substrate	Enzyme concentration	$C_1$	Relative rate
	mg protein N per ml		
Carbobenzoylglycyl-L-phenylalanine	0.00157	14	100
Carbobenzoylglycyl-L-tyrosine	0.00157	6.4	43
Carbobenzoylglycyl-L-histidine	0.157	0.07	0.5
“	0.079	0.07	0.5
Carbobenzoylglycyl-L-phenylalanine*	0.0039	4.1	100
Carbobenzoyl-L-histidyl-L-phenylalanine*†	0.0039	3.6	88
“	0.0078	4.1	100
Carbobenzoyl-L-histidyl-L-tyrosine*†	0.0039	2.8	68
“	0.0078	3.2	78
Carbobenzoyl-L-histidylglycine*	0.157	0.01	0.03

\* In 35 per cent methanol

† Substrate not completely soluble

the use of carboxypeptidase for the stepwise degradation of proteins and peptides

The rates of hydrolysis of substrates in which the carboxyl group of histidine is involved in peptide linkage are in accord with previous observations that the residue adjacent to the terminal amino acid has little effect on the susceptibility to carboxypeptidase. Both carbobenzoxy-L-histidyl-L-phenylalanine and carbobenzoxy-L-histidyl-L-tyrosine are excellent substrates and are hydrolyzed at essentially the same rate as carbobenzoxyglycyl-L-phenylalanine. Carbobenzoxy-L-histidylglycine, as expected from studies with other acylated dipeptides containing C-terminal glycine (17), is hydrolyzed very slowly

TABLE III

*Action of Carnosinase on Peptides Containing Histidine*

Carnosinase was prepared according to the directions of Hanson and Smith (1). Both the unactivated and  $Mn^{++}$ -activated (0.01 M  $Mn^{++}$ ) preparations were used at the levels indicated. Assays were made at 39° in 0.04 M Tris buffer at pH 8.0

Substrate	No activation, $E = 0.512$ mg protein N per ml $C_0$	$Mn^{++}$ -activated, $E = 0.38$ mg protein N per ml $C_0$	$\frac{C_0 \text{ (no activation)}}{C_0 \text{ (} Mn^{++} \text{ activated)}}$
Carnosine	0.98	2.5	0.39
Anserine	0.43	1.2	0.36
$\beta$ -Alanyl-L-histidylglycine*	1.03	2.4	0.43
L-Leucinamide		4.4	
L-Histidylglycine		2.5	

\* Both peptide bonds of this compound are hydrolyzed. The  $C_0$  values are calculated from the initial rate of hydrolysis.

**Carnosinase**—Swine kidney carnosinase was prepared as described by Hanson and Smith (1). Assays were made with both the unactivated and the  $Mn^{++}$ -activated preparations at pH 8.0 in the presence of 0.04 M Tris. The results are shown in Table III. The tripeptide,  $\beta$ -alanyl-L-histidylglycine, is hydrolyzed by the carnosinase preparation, however, from the extent of the hydrolysis, 160 per cent in 19 hours, and the hydrolysis of L-leucinamide and L-histidylglycine by the enzyme preparation, it is likely that there is a contaminating enzyme, probably leucine aminopeptidase, which is acting on the liberated dipeptide, L-histidylglycine. (As shown in Table I, the aminopeptidase cannot hydrolyze a compound with a  $\beta$  amino group.) This view is supported by the difference between the ratios of the  $C_0$  of the activated and unactivated enzymes toward this substrate and of carnosine and anserine.

The hydrolysis of anserine is noteworthy, since the close agreement of

the ratios of the proteolytic coefficients of the activated and unactivated preparations suggests that a single enzyme is responsible for the hydrolysis of anserine and carnosine. This poses the question whether the anserinase of fresh codling studied by Jones (18) will hydrolyze carnosine. A comparison of the properties of the two enzymes shows only slight differences and many similarities.

**Papain**—Crystalline mercuripapain was prepared by the method of Kimmel and Smith (19). Assays were performed in 0.02 M acetate buffer (pH 4.6 to 5.6) and in the presence of 0.005 M cysteine and 0.001 M Versene (19). Table IV shows that the several derivatives of histidine which were tested are rather poor substrates for papain when compared with benzoyl-

TABLE IV  
*Hydrolysis of L-Histidine Derivatives by Papain*

Crystalline mercuripapain prepared by the directions of Kimmel and Smith (19) was used at concentrations of 0.006 to 0.63 mg of protein N per ml, according to the substrate\*. The enzyme was made up in a solution containing 0.005 M cysteine and 0.001 M Versene to achieve full activation. Assays were made at 39° with 0.05 M substrate and 0.04 M sodium acetate buffer at the pH indicated.

Substrate	pH	C <sub>1</sub>	Relative rate
α-Benzoyl-L-argininamide	5.0	1.2	100
L-Histidinamide	4.6	0.01	0.8
Carbobenzoxy-L-histidyl-L-leucinamide	4.8	0.013	1.0
Carbobenzoxy-L-histidinamide	5.6	0.11	9.0

\* The preparation of the papain and the assays were performed by Dr. J. R. Kimmel.

argininamide, this is in accord with earlier results of Bergmann and Fruton (4).

**Chymotrypsin**—A commercial preparation (Armour and Company) of crystalline bovine chymotrypsin (Lot 90492) was used for this study. The substrates were present at a concentration of 0.05 M, but, because of their sparing solubility, were mainly in suspension throughout the experiment. The results, therefore, can only be considered as qualitative.

Table V shows that the histidine derivatives are rather poor substrates for chymotrypsin, carbobenzoxy-L-histidyl-L-tyrosinamide and carbobenzoxy-L-histidyl-L-phenylalaninamide being split at about one-tenth the rate and carbobenzoxy-L-histidyl-L-leucinamide and carbobenzoxyglycyl-L-histidinamide at about one twenty-fifth the rate of carbobenzoxyglycyl-L-tyrosinamide. The low susceptibility of these substrates emphasizes, as in the instance mentioned above with carboxypeptidase, that the uncharged

TABLE V

*Chymotrypsin Action on Derivatives of Histidine and Tryptophan*

Crystalline chymotrypsin was used at a concentration of 1.54 mg of protein \ per ml. Assays were made at 39° with 0.05 M substrate in 0.04 M Tris buffer, pH 8.0. All substrates were in suspension throughout the period of assay. The products of the reaction were identified by means of one-dimensional paper chromatography on Whatman No. 1 paper with butanol-acetic acid-water (200:30:75). Cbz = carbobenzoxy.

Substrate	Hydrolysis in 24 hrs  per cent	$k_1 \times 10^3$	Relative rate	Product identified		Negative test	
				Ninhydrin reaction	Pauly reaction	Ninhydrin reaction	Pauly reaction
Cbzglycyl-L-tyrosinamide	155	3.2	100	Tyrosine		Tyrosinamide	
Cbz-L-tryptophylglycinamide	86	1.6	50	Glycinamide		Glycine	
Cbzglycyl-L-tryptophanamide	55	1.2	38	Tryptophan		Tryptophanamide	
Cbz-L-histidyl-L-tyrosinamide	29	0.32	10		Cbzhistidyltyrosine	Tyrosine Tyrosinamide	
Cbz-L-histidyl-L-phenylalaninamide	33	0.24	8		Cbzhistidylphenylalanine	Phenylalanine (amide)	
Cbz-L-histidyl-L-leucinamide	30	0.12	4	Leucine			Cbzhistidylleucine
Cbzglycyl-L-histidinamide	16	0.11	3		Cbzglycylhistidine	Histidine + amide	Histidine + amide

imidazole grouping does not behave as an aromatic residue in these compounds.

After the enzyme had been allowed to act for 24 hours, the products were identified by one-dimensional paper chromatography on Whatman No. 1 paper with a butanol-acetic acid-water solvent (200:30:75) (Table

V) and use of the ninhydrin and Pauly reaction for location and identification of the reaction products. For the three substrates, carbobenzoxyglycyl-L-histidinamide, carbobenzoxy-L-histidyl-L-phenylalaninamide, and carbobenzoxy-L-histidyl-L-tyrosinamide, detectable hydrolysis had occurred only at the amide linkage. The three acylated dipeptides were definitely identified and no indication was obtained of the presence of free histidine, phenylalanine, or tyrosine, or their respective amides. The hydrolysis of carbobenzoxy-L-histidyl-L-leucinamide was different. Here, the cleavage occurred between histidine and leucine, as well as at the amide bond as indicated by the identification of leucine with no indication of the acylated dipeptide. This is somewhat unexpected in light of the behavior of the other histidine-containing substrates. Moreover, Sanger and Tuppy (20) did not detect any action of chymotrypsin on the two sequences of histidyl-leucine which occur in the oxidized B chain of insulin. With the exception of benzoyl-norleucine ethyl ester, which is hydrolyzed very slowly, and D-leucylglycine, D-leucylglycylglycine, benzoyl-L-leucyl-L-leucylglycine, and carbobenzoxyglycyl-L-leucylglycinamide, which are not hydrolyzed at all (21), derivatives of leucine have apparently not been investigated as substrates for chymotrypsin. It has also been reported that nicotinyl-D-histidinamide is not hydrolyzed by this enzyme (21).

The hydrolysis of the two tryptophan compounds was investigated, since only a few such compounds have been studied (21). From Table V it is evident that both carbobenzoxyglycyl-L-tryptophanamide and carbobenzoxy-L-tryptophylglycinamide are good substrates for chymotrypsin, the former compound being hydrolyzed at both the amide and peptide bonds and the latter at only the peptide bond.

### *Synthesis of Peptides and Derivatives*

*L-Histidine Methyl Ester Dihydrochloride*—Because the classical method (22) of esterifying histidine is somewhat troublesome, the following simplified procedure was developed. L-Histidine hydrochloride (30 gm) was esterified in 450 ml of absolute methanol with 8 ml of concentrated sulfuric acid under reflux for 1 hour and then for 2 hours with dry HCl. On cooling, the product crystallized as the dihydrochloride, yield, 33 gm, m p, 200–201°.

*Carbobenzoxy- $\beta$ -alanyl-L-histidine Hydrazide*—To 3.74 gm of crystalline carbobenzoxy- $\beta$ -alanyl-L-histidine methyl ester (23) in 50 ml of ethanol, 2 ml of 95 per cent hydrazine hydrate were added. After 3 hours at 50° the reaction mixture was cooled to 0° and the product was collected by filtration, washed with cold ethanol, and air-dried, yield, 3.0 gm, m p, 188–189°.

<sup>1</sup> Analyses for compounds containing histidine are given in Table VI and were performed by Weiler and Strauss of Oxford.

TABLE VI  
Analysis of Histidine Derivatives

Compound	Method	Yield per cent	Formula	Calculated			Found			M p °C
				C	H	N	C	H	N	
				per cent	per cent	per cent	per cent	per cent	per cent	
Cbz- $\beta$ -alanyl-L-histidinamide	A	70	$C_{17}H_{21}O_4N_5$	56.8	5.9	19.5	57.0	5.8	19.7	179-180
Cbz- $\beta$ -alanyl-L-histidine hydrazide		78	$C_{17}H_{22}O_4N_6$	54.6	5.9	17.1	55.0	5.9	17.0	188-189
Cbz- $\beta$ -alanyl-L-histidylglycine ethyl ester		51	$C_{21}H_{27}O_6N_5$	56.6	6.1	15.7	55.8	5.9	16.1	154-156
Cbz- $\beta$ -alanyl-L-histidylglycine	B	76	$C_{19}H_{23}O_6N_5$	54.6	5.5	16.8	55.6	5.5	16.6	166-167
$\beta$ -Alanyl-L-histidylglycine $HNO_3$		66	$C_{11}H_{17}O_4N_5 \cdot HNO_3$	38.2	5.2	24.2	38.0	5.5	24.0	219-220
Cbz-glycyl- $\beta$ -alanyl-L-histidine methyl ester		51	$C_{20}H_{25}O_6N_5$	55.7	5.8	16.3	55.9	5.8	16.0	168-169
Cbz- $\beta$ -alanyl-L-histidyl-L-histidine methyl ester $H_2O$	A	20	$C_{24}H_{29}O_6N_7 \cdot H_2O$	55.0	6.1	18.3	54.5	5.9	18.5	160-161
Cbz-L-histidinamide	A	20	$C_{20}H_{20}O_3N_4$	66.0	5.5	15.4	66.0	5.7	15.0	174-175
Cbz-L-histidinamide $\frac{1}{2}H_2O$		81	$C_{14}H_{16}O_3N_4 \cdot \frac{1}{2}H_2O$	56.7	6.0	18.8	57.1	5.9	18.8	196-197
L-Histidinamide $2HCl$ *		64	$C_6H_{10}ON_4 \cdot 2HCl$	31.8	5.3	23.6	32.1	5.4	23.6	260-261
Cbz-L-histidylglycine benzyl ester	A	80	$C_{23}H_{24}O_6N_4$	63.2	5.6	12.9	62.7	5.7	13.1	93-94
L-Histidylglycine $HCl \cdot \frac{1}{2}H_2O$ †		81	$C_8H_{12}O_3N_4 \cdot HCl \cdot \frac{1}{2}H_2O$	37.4	5.5	21.7	37.9	5.5	21.6	229-230
Cbz-L-histidyl-L-phenylalanine methyl ester		53	$C_{24}H_{28}O_6N_4$	64.0	5.8	12.5	63.8	5.8	12.7	157-158
Cbz-L-histidyl-L-phenylalanine	A	92	$C_{23}H_{24}O_6N_4$	63.4	5.5	12.9	63.4	5.8	12.9	230-231
L-Histidyl-L-phenylalanine $HCl$		26	$C_{18}H_{18}O_3N_4 \cdot HCl \cdot H_2O$	50.2	5.9	15.7	50.2	5.9	15.1	193-194
Cbz-L-histidyl-L-tyrosine ethyl ester		36	$C_{25}H_{28}O_6N_4$	62.5	5.9	11.7	62.2	5.9	11.6	94-95
Cbz-L-histidyl-L-tyrosine $\frac{1}{2}H_2O$	B	22	$C_{23}H_{24}O_6N_4 \cdot \frac{1}{2}H_2O$	60.0	5.4	12.1	60.0	5.4	11.8	232-233
Cbz-L-histidyl-L-alanine ethyl ester		27	$C_{19}H_{22}O_5N_4$	58.8	6.2	11.1	59.2	6.6	11.2	122-123
Cbz-L-histidyl-L-histidine methyl ester		28	$C_{21}H_{24}O_6N_6$	57.1	5.5	19.0	57.1	5.7	18.9	71-75

Cbz-L-histidyl-L-leucine methyl ester	A	48	$C_{21}H_{28}O_6N_4$	60 5	6 8	13 4	60 6	6 7	13 1	121-122
Cbz-L-histidyl-L-hydroxyproline methyl ester	B	36	$C_{20}H_{24}O_6N_4$	57 8	5 8	13 5	57 8	5 8	14 0	75-76
Cbz-L-histidyl-L-leucinamide		62	$C_{20}H_{27}O_4N_5$	60 0	6 8	17 1	60 2	6 9	17 3	183-184
Cbz-L-histidylglycylglycine ethyl ester	B	70	$C_{20}H_{25}O_6N_5$	55 6	5 9	16 2	55 6	5 9	16 2	180-181
Cbz-L-histidylglycine ethyl ester	A	75	$C_{18}H_{21}O_5N_4$	58 0	5 7	15 1	58 5	5 8	14 9	113-114
Cbz-L-histidyl-L-phenylalaninamide		80	$C_{23}H_{25}O_4N_5$	63 4	5 8	16 1	63 3	5 6	15 9	203-204
Cbz-L-histidyl-L-tyrosinamide $\frac{1}{2}H_2O$		73	$C_{23}H_{25}O_6N_5 \cdot \frac{1}{2}H_2O$	60 1	5 7	15 2	60 5	5 6	15 3	205-206

Cbz = carbobenzoyl

\*  $[\alpha]_D^{20} +22.0$  (1 per cent,  $H_2O$ )†  $[\alpha]_D^{20} -8.43$  (1 per cent,  $H_2O$ )‡  $[\alpha]_D^{20} -2.05$  (1 per cent,  $H_2O$ )



Carbobenzoyglycyl- $\beta$ -alanine ethyl ester (24) was converted to the corresponding hydrazide by the same procedure, m p, 142–143°

$C_{13}H_{18}O_4N_4$ (294)	Calculated	C 53.0, H 6.1, N 19.1
	Found	" 53.0, " 6.2, " 19.2

The preparation of carbobenzoy-L-histidine hydrazide (10) has been described

*Azide Couplings*—The transformation of the hydrazides to the azides and their reaction with amino acid esters were accomplished by two general procedures which are given below for representative cases. Method A was preferred because of convenience. Method B was used only for the amino acid or peptide esters which were too soluble in the aqueous phase to give good yields by Method A.

*Method A Carbobenzoy-L-histidylglycine Benzyl Ester*—3.03 gm of carbobenzoy-L-histidine hydrazide in 100 ml of 0.24 N HCl were converted to the azide at 0° by the action of 1.24 gm of  $NaNO_2$ . After 2 minutes glycine benzyl ester hydrochloride (2.1 gm) and triethylamine (28 ml) in 50 ml of cold chloroform were added with vigorous stirring. After stirring for 30 minutes in the cold, the chloroform layer was separated, washed with water, and dried over  $Na_2SO_4$ . The dried solution was concentrated to dryness and the residue was dissolved in ether. On treatment with petroleum ether the product precipitated, yield, 3.5 gm, m p, 134–136°.

*Method B Carbobenzoy-L-histidylglycylglycine Ethyl Ester*—3.03 gm of carbobenzoy-L-histidine hydrazide in 15 ml of 2 N HCl were converted to the azide at 0° by the addition of 0.7 gm of  $NaNO_2$ . After 2 minutes, 50 ml of cold chloroform containing 2.8 ml of triethylamine were added. After equilibration, the chloroform solution of the azide was separated and dried over  $Na_2SO_4$ . The dried azide solution was added to a cold chloroform solution containing 1.97 gm of glycylglycine ethyl ester hydrochloride and 1.4 ml of triethylamine. On standing overnight the product crystallized, yield, 3.0 gm, m p 180–180.5°.

If the product of the reaction was soluble, the reaction mixture was washed in the usual manner and the solvent was removed by concentration. Trituration of the residue with petroleum ether was very effective for crystallization.

*Amides and Hydrazides*—All hydrazides were prepared as described above. The amides were prepared by the usual procedure by employing alcoholic ammonia except for carbobenzoy-L-histidinamide. Direct amidation of L-histidine methyl ester dihydrochloride has been described (9), but the yield was poor. Treatment of carbobenzoy-L-histidine azide (prepared as described above) with a 3-fold excess of  $NH_4OH$  yielded

the corresponding amide in good yield. Similarly, reaction of the azide with aniline produced the anilide (11), although in lower yield. Reduction of carbobenzoxy-L-histidinamide by the usual procedure gave pure L-histidine amide dihydrochloride in excellent yield.

### Saponification

*Carbobenzoxy- $\beta$ -alanyl-L-histidylglycine*—4 ml of 1 N NaOH were added to 1.4 gm of the carbobenzoxytripeptide ester in 10 ml of 50 per cent dioxane. After 18 hours the mixture was filtered and the filtrate was adjusted to pH 5.8 with 0.8 ml of 5 N  $\text{H}_2\text{SO}_4$ . After concentration to dryness *in vacuo*, the residue was extracted with hot ethanol ( $3 \times 15$  ml). The product crystallized from the combined filtrates on cooling, yield, 1.0 gm, m p, 166–167°.

*Carbobenzoxy-L-histidyl-L-phenylalanine*—1.5 gm of carbobenzoxy-L-histidyl-L-phenylalanine methyl ester in 15 ml of methanol were saponified with 1.1 equivalents of 1 N NaOH for 1 hour. 1.1 equivalents of 1 N HCl and 10 ml of water were added and the product crystallized immediately, yield, 1.6 gm, m p, 230–230.5°.

Carbobenzoxy-L-histidyl-L-tyrosine was prepared in the same way. All other saponified products did not precipitate on neutralization and were very slow to crystallize. Only carbobenzoxy-L-histidyl-L-leucine and carbobenzoxy-L-histidyl-L-alanine, both of which have been described (10), could be obtained analytically pure.

*Reduction*—All reductions were performed in the usual manner in methanol and in the presence of the calculated equivalent of HCl. The reduced compounds were crystallized from water-methanol or water-acetone.

### Tryptophan Derivatives

*Carbobenzoxy-L-tryptophylglycine Ethyl Ester*<sup>2</sup>—Carbobenzoxy-L-tryptophan (6.8 gm) (25) was converted to the acid chloride in ethyl acetate and coupled with glycine ethyl ester prepared from 5.6 gm of the hydrochloride. After 1 hour, the preparation was filtered, washed in the usual manner, and dried over sodium sulfate. The solution was concentrated to a thick syrup which was allowed to stand under ether, yield, 5.2 gm of needles, m p, 120°.

$\text{C}_{23}\text{H}_{25}\text{O}_5\text{N}_3$  (423.4) Calculated, N 9.9, found, N 9.7

*5-Indolylhydantoin-3-acetamide*<sup>2</sup>—The above ester (3.9 gm) was treated for 2 days at room temperature with 50 ml of methanol which had been previously saturated with dry ammonia at 0°. The product was obtained

<sup>2</sup> This compound was prepared by Dr. Emil L. Smith.

by repeated concentration *in vacuo* with methanol and crystallized with ether. After recrystallization from hot methanol, the melting point was 196°. Analysis indicated that the substance was the hydantoin named above instead of the expected carbobenzoxy dipeptide amide. Similar hydantoin has been obtained as products of the reaction of dry ammonia and the corresponding carbobenzoxy dipeptide esters of phenylalanine, leucine, and methionine (26).

$C_{14}H_{14}O_3N_4$ (286.2)	Calculated	C 59.4, H 4.9, N 19.6
	Found	" 59.2, " 5.1, " 20.0

*Carbobenzoxy-L-tryptophylglycinamide*—To 3.38 gm. of carbobenzoxy-L-tryptophan in 2 ml. of dioxane, 1.4 ml. of triethylamine were added at 0°, followed by 2 ml. of isobutylchlorocarbonate. After 10 minutes, 1.1 gm. of glycine hydrochloride in 5 ml. of 2 N NaOH were added and the mixture was stirred for 2 hours. The product, an oil, was extracted with chloroform and, after being washed in the usual manner and after removal of the solvent *in vacuo*, was crystallized by repeated concentration from ether, yield, 2.0 gm., m.p., 135–136°.

$C_{21}H_{22}O_4N_4$ (394.4)	Calculated	C 64.0, H 5.6, N 14.2
	Found	" 64.2, " 5.7, " 14.3

The author wishes to express his appreciation to Dr. E. L. Smith for his advice and suggestions during the course of this investigation.

#### SUMMARY

1. A number of peptides and peptide derivatives containing histidine and some containing tryptophan have been synthesized and tested as substrates for certain proteolytic enzymes.

2. Leucine aminopeptidase hydrolyzes L-histidinamide and peptides containing the carboxyl group of histidine in peptide linkage. No hydrolysis of carnosine was detected and glycyl-L-histidine is hydrolyzed slowly.

3. Crystalline carboxypeptidase hydrolyzes carbobenzoxy dipeptides in which histidine is in the *N*-terminal position, however, carbobenzoxy glycyl-L-histidine was split at only 0.005 times the rate of carbobenzoxy glycyl-L-phenylalanine, the best known substrate for this enzyme.

4. Carnosinase hydrolyzes anserine at about half the rate of carnosine and also splits  $\beta$ -alanyl-L-histidylglycine.

5. L-Histidinamide, carbobenzoxy-L-histidinamide, and carbobenzoxy L-histidyl-L-leucinamide are relatively poor substrates for crystalline papain.

6. The action of crystalline chymotrypsin on several carbobenzoxy dipep-

tide amides was also studied and the point at which hydrolysis occurred was established by means of paper chromatography. The histidine-containing compounds were found to be poor substrates for this enzyme. Carbobenzoyglycyl-L-tryptophanamide and carbobenzoy-L-tryptophylglycinamide proved to be excellent substrates for chymotrypsin, the former compound being hydrolyzed at both the peptide and amide bonds and the latter only at the peptide bond.

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# THE INHIBITION OF ASPARTIC ACID UTILIZATION IN THE SYNTHESIS OF THE ADAPTIVE "MALIC ENZYME" IN *LACTOBACILLUS ARABINOSUS*

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The inhibition of utilization of amino acids in the synthesis of adaptive enzymes by amino acid analogues has been the subject of several recent studies (1-3). In most instances, it has been possible to demonstrate that the inhibition could be reversed by addition of competitive quantities of the corresponding amino acid. In the present investigation, the inhibitory effects of the aspartic acid analogues, cysteic acid and  $\beta$ -hydroxyaspartic acid, on the synthesis of the adaptive "malic enzyme" by *Lactobacillus arabinosus* 17-5 (4) have been studied, and the abilities of asparagine, glycylasparagine, and aspartic acid to reverse these inhibitions have been determined. The study demonstrates that more than one site of inhibition by the analogues exists, and that the peptide and asparagine are utilized by routes which do not involve the free amino acid.

## EXPERIMENTAL

Cells of *L. arabinosus* 17-5 were taken from a stock culture maintained on yeast extract-glucose agar and grown on 8 ml of yeast extract-peptone medium (5) for 16 hours at 30°. 1 ml of this suspension was transferred to 8 ml of the medium described above, and growth was allowed to continue for an additional 8 hours. The cells were collected by centrifugation, washed once with 10 ml of distilled water, and resuspended in 3 ml of distilled water. 2 drops of this heavy suspension were used to inoculate 80 ml of a synthetic medium similar to one previously described (6), in a 250 ml Erlenmeyer flask provided with a cotton plug. Each liter of the growth medium contained 20 mg of L- or 40 mg of DL-amino acids,<sup>1</sup> B vitamins,<sup>2</sup> 2 ml, inorganic Salts A and B (7), 5 ml each, sodium acetate,

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<sup>1</sup> The complete amino acid mixture consisted of DL-alanine, L-arginine hydrochloride, L-aspartic acid, L-cysteine hydrochloride, L-glutamic acid, glycine, L-histidine, L-hydroxyproline, DL-isoleucine, L-leucine, DL-lysine hydrochloride, DL-methionine, L-phenylalanine, L-proline, DL-serine, DL-threonine, L-tryptophan, L-tyrosine, and L-valine obtained from commercial sources.

<sup>2</sup> The B vitamin solution contained thiamine hydrochloride, 3 mg, nicotinic acid, 3 mg, calcium pantothenate, 3 mg, riboflavin, 3 mg, pyridoxine hydro-

6 gm, uracil, adenine sulfate, and guanine hydrochloride, 4 mg each, ammonium chloride, 3 gm, and glucose, 10 gm. After incubation at 30° for 16 hours, the cells (25 to 30 mg, dry weight, of cells per 80 ml of growth medium) were harvested and washed twice with 25 ml portions of cold distilled water, so as to provide a suspension of 1.5 mg of cells per ml (on a dry weight basis as determined by the use of a turbidimeter and a standard curve of mg of cells *versus* galvanometer deflection). A fresh cell suspension was prepared each day.

The rate and extent of "malic enzyme" synthesis were determined by a procedure similar to that described by Blanchard *et al* (4). Carbon dioxide evolved by the action of the enzyme on malic acid was determined manometrically by use of the Warburg technique. The basal medium consisted of DL-malic acid, 240  $\mu$ moles, glucose, 120  $\mu$ moles, magnesium chloride, 0.06  $\mu$ mole, manganese chloride, 0.06  $\mu$ mole, and potassium phosphate buffer, pH 5.5, 70  $\mu$ moles. The main chamber of the control flask contained supplements of adenine sulfate, guanine hydrochloride, and uracil, 20  $\gamma$  each, 0.1 ml of a 1:20 dilution of Salts B, sodium acetate, 7  $\mu$ moles, 0.1 ml of a 1:10 dilution of the vitamin solution,<sup>2</sup> and either 20  $\gamma$  per ml of the L form or 40  $\gamma$  per ml of the DL form of each of the amino acids listed in the complete amino acid mixture except aspartic acid, which was added as indicated in the various experiments.

L-Cysteic acid,<sup>3</sup> prepared by the method of Gortner and Hoffman (8), and DL- $\beta$ -hydroxyaspartic acid,<sup>3</sup> prepared by the method of Dakin (9) as extensively modified by Skinner and Shive,<sup>4</sup> were added to the main chamber in solutions of appropriate concentrations. Aspartic acid, L-asparagine, and glycyl-L-asparagine were added as indicated in various experiments to the main chamber. All solutions were adjusted to pH 5.5 before addition to the flasks. The constituents in the main chamber of each flask were diluted to a volume of 2.6 ml. The flasks and their contents were chilled in a cracked ice bath, and the chilled cell suspension containing 0.6 mg of dry weight of cells was added to the side arm of each flask to provide a total volume of 3 ml. The system was then flushed with nitrogen, during which time the flasks were chilled by intermittent immersion in ice water. After temperature equilibration at 37°, the reaction was initiated by tipping the cells into the main compartment.

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chloride, 50 mg, inositol, 15 mg, biotin, 15  $\gamma$ , *p*-aminobenzoic acid, 150  $\gamma$ , and folic acid, 150  $\gamma$ , in 30 ml of 50 per cent alcohol.

<sup>2</sup> The authors are indebted to Dr. C. G. Skinner for the cysteic acid and hydroxyaspartic acid used in this work. The less soluble of the two diastereoisomers of hydroxyaspartic acid, termed "para"-hydroxyaspartic acid by Dakin (9), was used in this work.

<sup>4</sup> Skinner, C. G., and Shive, W., unpublished data.

The amount of carbon dioxide evolution was measured at 15 minute intervals, and the results were expressed as per cent of carbon dioxide evolution of the control in the 1st hour of incubation. There is no detectable cell multiplication during this period, as determined by plating experiments. The control incubation medium is optimal in all respects, except amino acid concentration, which was maintained at low levels to allow studies of amino acid antagonisms over broad ranges of concentrations. Carbon dioxide evolution during the 1st hour averages about 180  $\mu$ l, whereas, under similar conditions with adequate concentrations of acid-hydrolyzed casein furnishing higher concentrations of amino acids, an increase in carbon dioxide evolution of 2-fold or slightly more may be obtained.

Occasionally, cell preparations were obtained which were somewhat resistant to inhibition by cysteic acid but not by hydroxyaspartic acid. A 2 hour depletion period, during which the cells were incubated in the growth medium lacking in exogenous aspartic acid, restored sensitivity to inhibition by cysteic acid without affecting inhibition by hydroxyaspartic acid. Thus, a reversing agent for cysteic acid but not hydroxyaspartic acid accumulates in the cells. Since the agent is depleted in the absence of exogenous aspartic acid but not in its presence, it appears likely that it is a derivative of aspartic acid. The relative inhibitory activities of hydroxyaspartic acid and cysteic acid varied several-fold in different experiments as a result of this variation in sensitivity to cysteic acid. In order to insure the validity of data which were obtained on different days with different cell preparations, it was necessary to repeat the experiments, and use only the experimental results in which quantitatively similar data were obtained so that identical controls in the different experiments gave essentially identical results. This was particularly necessary in comparative studies of the effectiveness of various reversing agents.

#### RESULTS AND DISCUSSION

The omission of aspartic acid from the incubation mixture causes a marked decrease in the amount of malic enzyme formed (Table I). The requirement for aspartic acid is satisfied almost as well by glycylasparagine, but asparagine is required in considerably larger quantities to promote a given amount of enzyme formation. Glycylaspartic acid was inactive. These results indicate either that asparagine and glycylaspartic acid are not readily absorbed by the cells or that the effectiveness of glycylasparagine cannot be attributed to its hydrolysis to free asparagine or to glycylaspartic acid. That the relatively small amount of enzyme activity found when aspartic acid is omitted from the incubation medium is not due to the presence of preformed malic enzyme is demonstrated by the finding that this activity could be further decreased by about 50 per cent by the



TABLE I

*Reversal of Cystic Acid Inhibition of Malic Enzyme Formation by Aspartic Acid, Glycylasparagine, and Asparagine*

L-Cysteic acid	Units reversing agent per ml *	L Aspartic acid	Glycyl-L-asparagine	L-Asparagine
		Enzyme activity, per cent of control†		
<i>mg per ml</i>				
None	0	17	17	17
	0 2	25		
	0 5	40	28	
	1	60	41	
	2	77	63	18
	5	94	90	26
	10	95	100	41
	20	100	103	60
	50			91
	100			106
0	0	13	13	17
0 2	0	6	6	9
	2	19	21	
	5	27	46	
	10	43	69	
	20	57	73	21
	50	73	79	40
	100	78		64
	200			81
	500	96	79	86
	1,000			87
0	0	13	20	13
2	0	6	11	6
	2		18	
	5		31	
	10	14	56	
	20	20	71	
	50	43	77	34
	100	65		45
	200	72		66
	500	83		72
	1,000	91	77	74
	10,000			76
0	0	10	17	13
20	0	5	9	6
	5		25	
	10		47	
	20		57	
	50		67	17

TABLE I—*Concluded*

L Cysteic acid  mg per ml	Units reversing agent per ml *	L Aspartic acid	Glycyl L asparagine	L Asparagine
		Enzyme activity, per cent of control†		
20	100			25
	200	28		38
	500	50	67	61
	1,000	63		63
	10,000	85	61	61

\* 1 unit of reversing agent is the molar equivalent of 1  $\gamma$  of L-aspartic acid

† Per cent of carbon dioxide evolution of the control, containing 20  $\gamma$  of L-aspartic acid per ml of complete amino acid mixture and no inhibitor, in the 1st hour of incubation in the presence of malic acid

addition of as little as 200  $\gamma$  per ml of cysteic acid. Since this effect is observed in the absence of exogenous aspartic acid, so that any aspartic function in malic enzyme synthesis must be performed by an endogenous source of aspartic acid, the site of action of cysteic acid under these conditions is not on the process of absorption of aspartic acid into the cell, but involves an inhibitory effect within the cell. Previous evidence for an internal site of action of cysteic acid has been obtained in growth inhibition studies with *Leuconostoc dextranicum* under conditions such that the organism was forced to synthesize its own aspartic acid (10).

The reversal of cysteic acid inhibition of enzyme synthesis by aspartic acid appears to be of the competitive type over a range of concentrations (Table I). On the other hand, only a 2-fold increase in concentration of glycylasparagine is required for reversal of inhibition by cysteic acid over the range from 0.2 to 20 mg per ml. However, in contrast to reversal with aspartic acid, the reversal of inhibition with glycylasparagine is never complete and becomes less complete at higher concentrations of cysteic acid, so that only 67 per cent reversal is attained at 20 mg per ml of cysteic acid. The decreasing effectiveness of glycylasparagine in restoring maximal synthesis with increasing inhibitor concentrations suggests that, if it were possible to use higher concentrations of inhibitor, glycylasparagine probably could be rendered ineffective as a reversing agent in contrast to the truly competitive reversing effect of aspartic acid. Glycylasparagine apparently furnishes an "active aspartic acid" through a process not involving the free amino acid and bypasses the major site of cysteic acid inhibition, but at very high concentrations cysteic acid can apparently inhibit the utilization of the peptide in some manner, probably at some metabolic site other than the initial site of utilization. Thus, under certain conditions, the free amino acid is best utilized for enzyme

synthesis, and under other conditions the peptide is the more effectively utilized

The effect of asparagine on cysteic acid inhibition of enzyme synthesis is similar to that of glycylasparagine, except that it is considerably less effective (Table I). Since a certain amount of asparagine is probably required for incorporation into the protein, the effect of asparagine on the amount of aspartic acid necessary for reversal of the inhibition was determined as indicated in Table II. Asparagine at a concentration of

TABLE II  
*Effect of Asparagine on Aspartic Acid Reversal of Cysteic Acid  
Inhibition of Malic Enzyme Formation*

L-Cysteic acid	L-Aspartic acid	Supplements	
		None	L-Asparagine, 10 $\gamma$ per ml <sup>*</sup>
		Enzyme activity, per cent of control <sup>a</sup>	
mg per ml	$\gamma$ per ml		
2	0		13
	10	32	43
	20	49	62
	50	77	
20	0		8
	50	23	30
	100	39	48
	200	52	

\* Per cent of carbon dioxide evolution of the control, containing 20  $\gamma$  of L aspartic acid per ml of complete amino acid mixture and no inhibitor, in the 1st hour of incubation in the presence of malic acid

10  $\gamma$  per ml decreases 2-fold the amount of aspartic acid required to reverse either 2 or 20 mg per ml of cysteic acid. Since asparagine exerts such a sparing effect, it appears that cysteic acid also prevents the synthesis of asparagine for incorporation into the enzyme. Since asparagine at high concentrations can also replace all of the functions of aspartic acid, a mechanism for its utilization other than by formation of the free amino acid must also exist, however, this route is less efficient than that for utilization of glycylasparagine.

Inhibition of malic enzyme synthesis by hydroxyaspartic acid is also competitively reversed by aspartic acid. The results (Table III) are qualitatively comparable to those obtained with cysteic acid, but hydroxyaspartic acid was found to be less effective as an inhibitor than cysteic acid.

TABLE III

*Reversal of Hydroxyaspartic Acid Inhibition of Malic Enzyme Synthesis  
by Aspartic Acid, Glycylasparagine, and Asparagine*

DL-Hydroxyaspartic acid*	Units reversing agent per ml †	L-Aspartic acid	Glycyl L asparagine	L-Asparagine
		Enzyme activity, per cent of control‡		
mg per ml				
0	0	17	17	17
0.2	0	9	9	10
	1	36	20	
	2	62	41	
	5	90	74	
	10	95	75	
	20	98		19
	50			32
	100		96	70
	200		90	92
	500		96	97
				104
0	0	12	12	12
2	0	8	8	8
	2	17	28	
	5	40	53	
	10	59	64	
	20	74	66	18
	50	92	71	42
	100	95		65
	200	103		72
	500			88
	1,000		71	100
0	0			
20	0			
	2		21	
	5		45	
	10		62	
	20	25	65	
	50	50	69	30
	100	64		45
	200	70		63
	500	90		69
	1,000	99	68	76
	10,000			74

\* "Para" isomer

† 1 unit of reversing agent is the molar equivalent of 1  $\gamma$  of L-aspartic acid

‡ Per cent of carbon dioxide evolution of the control, containing 20  $\gamma$  of L aspartic acid per ml of complete amino acid mixture and no inhibitor, in the 1st hour of incubation in the presence of malic acid

In order to determine whether hydroxyaspartic acid and cysteic acid inhibit the same or different sites involved in the utilization of aspartic acid for enzyme synthesis, a study of the effects of combinations of the two inhibitors over a range of concentrations was undertaken. The results are indicated in Fig 1, which also contains the theoretical results which

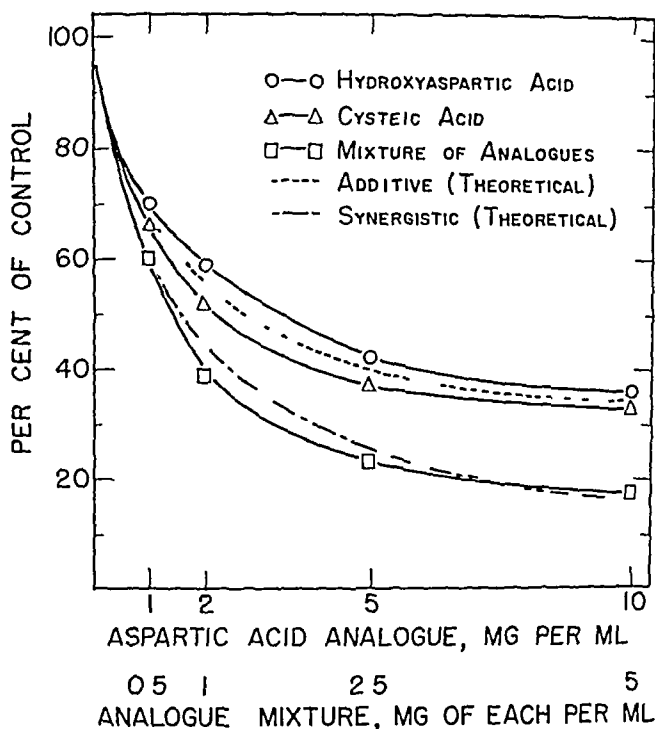


FIG 1 Inhibition of "malic enzyme" synthesis by combinations of cysteic acid and hydroxyaspartic acid. The cells were incubated for 2 hours in complete growth medium without exogenous aspartic acid, 0.8 mg of dry weight of cells per flask. The results are expressed as per cent of carbon dioxide evolution of the control (containing 20  $\gamma$  of L- or 40  $\gamma$  of DL-amino acids per ml, to provide the complete amino acid mixture, in the absence of inhibitor) in the first 90 minutes of incubation in the presence of malic acid. All the flasks contain L-aspartic acid, 20  $\gamma$  per ml. Other conditions are as described in the text.

would be expected if the two inhibitors were acting at the same site. The response to a combination of the inhibitors is not additive but approximates the product of the responses, expressed as per cent of controls, obtained separately with each inhibitor. The experimental and theoretical synergistic effects are shown in Fig 1. Such a synergistic effect could have been produced equally well by inhibition of two reactions in sequence or by independent inhibitions of separate sites on a template or macromolecule which is specific for the aspartate moiety. The latter mechanism would also require the utilization of glycylasparagine at these separate

sites presumably by a "transferase" type reaction. Evidence for the accumulation of an aspartic derivative capable of reversing primarily cysteic acid inhibition tends to favor the former mechanism. Further studies concerning this aspect of the problem are in progress.

A study of the reversal of the inhibition produced by a combination of cysteic acid and hydroxyaspartic acid by aspartic acid, glycylasparagine, and asparagine revealed no significant differences from the reversals obtained when each inhibitor was tested separately.

#### SUMMARY

Inhibitions of synthesis of the adaptive "malic enzyme" of *Lactobacillus arabinosus* 17-5 by the aspartic acid analogues, cysteic acid and  $\beta$ -hydroxyaspartic acid, are reversed competitively by aspartic acid. Glycylasparagine and, less effectively, asparagine reverse the inhibitions in a non-competitive manner, but do not completely reverse the inhibition at high concentrations of inhibitor. Since aspartic acid is slightly more effective than glycylasparagine in promoting enzyme synthesis in the absence of the inhibitor, but less effective in the presence of moderate concentrations of the aspartic acid antagonists, the peptide is utilized by a route not involving the free amino acid, but probably involving a common active intermediate. Cysteic acid and hydroxyaspartic acid are synergistic in inhibiting aspartic acid utilization. These results are compatible with a mechanism of inhibition of aspartic acid utilization either at two successive sites of a sequence of reactions or at independent sites on a template or macromolecule. The latter mechanism would also necessitate the utilization of glycylasparagine at these sites, presumably by a "transferase" type reaction.

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# A STUDY OF GROWTH INHIBITIONS BY THIENYLPYRUVIC ACID AND RELATED COMPOUNDS

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The ability of many organisms to use certain keto acids in the place of the corresponding amino acids is now well known (1-5). The utilization of a keto acid is often related to the ease with which it can enter into transamination reactions to form the corresponding amino acid. Jacquez *et al* (6) reported the preparation of thienylpyruvic acid and described studies in which it entered into certain transamination reactions in mouse and rat tissues. Meister (7) found that extracts from *Lactobacillus arabinosus* catalyzed transamination between  $\beta$ -2-thienylalanine and  $\alpha$ -ketoglutarate.

In view of the metabolic importance of keto acids, a study of the ability of phenylpyruvic acid to form phenylalanine and to prevent the toxicity of  $\beta$ -2-thienylalanine and its peptides was undertaken with *Escherichia coli* 9723 as the test organism. In addition, the ability of 2-thienylpyruvic acid to inhibit growth of the organism and the effects of phenylpyruvic acid and phenylalanine on the inhibition were studied. The possibility of the existence of separate sites of competition of amino acid, keto acid, and peptide and their corresponding analogues was also investigated.

## EXPERIMENTAL

**Compounds**—2-Thienylpyruvic acid was prepared as previously reported (6), except that in our procedure the azlactone intermediate was obtained from chloroacetyl- $\beta$ -2-thienylalanine (8). The melting point of thienylpyruvic acid was 164-165° (on a preheated block), confirming that reported by Jacquez *et al* (6). Other compounds were obtained commercially or were prepared by well known procedures.

**Testing Methods**—The assay procedures and medium for growth experiments have been previously described (9).

**Resting Cell Experiments**—For studies concerning the conversion of phenylpyruvic acid to phenylalanine, *E. coli* 9723 was grown overnight in

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the salts-glucose medium. The cells were centrifuged and then washed with saline and suspended in phosphate buffer at pH 7. 1 ml of cell suspension, when combined with 1 ml of solution of supplements, gave a final cell concentration of about 2 mg per ml. The tubes were incubated at 37° with occasional shaking. At intervals of 0, 60, 120, and 240 minutes, 10  $\mu$ l samples of the mixtures were withdrawn and placed on Whatman filter paper for preparation of the chromatograms. The sample was chromatographed by the ascending technique in 1-butanol saturated with water as the solvent, and the amino acids were detected by reaction with ninhydrin.

#### RESULTS AND DISCUSSION

On ninhydrin-treated chromatograms of the medium from incubated resting cells of *E. coli*, prepared as indicated above, a purple spot corresponding to phenylalanine appeared from medium containing phenylpyruvic acid with tryptophan, tyrosine, glutamic acid, thienylalanine, or ammonium chloride. Thienylpyruvic acid was similarly converted into thienylalanine, and the spots corresponding to phenylalanine and thienylalanine formed from the corresponding keto acids became more intense after incubations of 2 to 4 hours. The chromatogram represented in Fig. 1 was prepared from cell preparations which had been incubated for 2 hours with 0.5 mg of each of the compounds indicated. Control experiments showed that phenylpyruvic acid was not converted into phenylalanine by resting cells under these conditions in the absence of the nitrogen sources. Also, the cells alone did not yield detectable amounts of phenylalanine when suspended in the phosphate buffer alone, within the 4 hour period.

From the chromatogram, it is apparent that resting cells of *E. coli* 9723 can readily convert phenylpyruvic acid into phenylalanine when any one of a variety of amino acids is present, including thienylalanine, tryptophan, tyrosine, and glutamic acid. Unexpectedly, it was found that phenylpyruvic acid was rapidly converted into phenylalanine when ammonium chloride was present as the only nitrogen source. Similarly, thienylpyruvic acid was converted into thienylalanine in the presence of ammonium chloride as well as the amino acids. Since ammonium ion was found to be almost twice as effective as glutamic acid or other amino acids as a nitrogen source in the conversion of phenylpyruvic acid to phenylalanine (Fig. 1), a hitherto unrecognized amination mechanism apparently occurs in this strain of *E. coli*. Further studies which show that phenylpyruvic acid is converted to phenylalanine by a specific triphosphopyridine nucleotide-dependent dehydrogenase system are being published elsewhere.<sup>1</sup>

<sup>1</sup> Olvard, J., Dunn, F. W., and Shive, W., manuscript submitted for publication.

In growth assays, phenylpyruvic acid at low concentrations does not appreciably affect the toxicity of thienylalanine and its peptides for *E coli*. Even at a concentration of 5  $\gamma$  per 5 ml, the keto acid exerts only moderate effects, however, at higher concentrations phenylpyruvic acid non-competitively reverses the toxicity of peptides of  $\beta$ -2-thienylalanine (Table I). Phenylalanine has similarly been shown to reverse the toxicity of these peptides in the same manner (8).

In reversing the toxicity of  $\beta$ -2-thienylalanine, phenylpyruvic acid is less effective than phenylalanine at low concentrations of inhibitor, but

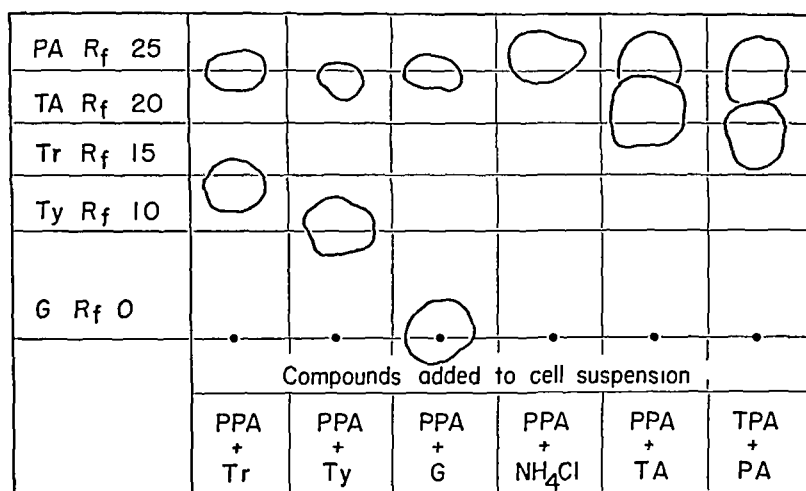


FIG 1 Drawing of typical chromatograms showing the conversion of phenylpyruvic acid and thienylpyruvic acid into phenylalanine and thienylalanine, respectively, in the presence of resting cells of *E coli* 9723. Abbreviations PA, phenylalanine, TA,  $\beta$ -2-thienylalanine, Tr, tryptophan, Ty, tyrosine, G, glutamic acid, PPA, phenylpyruvic acid, TPA, 2-thienylpyruvic acid.

becomes more effective than the amino acid at high concentrations of thienylalanine (Table II). Thus, only 5  $\gamma$  of thienylalanine inhibit growth in the presence of 5  $\gamma$  of phenylpyruvic acid in 5 ml cultures, while 1 mg of thienylalanine is required for growth inhibition in the presence of an equivalent amount of phenylalanine (10  $\gamma$  of the racemic form), but 50  $\gamma$  of phenylpyruvic acid reverse the growth inhibition by 5 mg of thienylalanine, which is not reversed by 100  $\gamma$  of DL-phenylalanine.

From initially low concentrations, an increase in the phenylpyruvic acid concentration requires more than a proportional increase in the thienylalanine concentration to effect the same degree of inhibition, but at moderate concentrations the relationship becomes competitive.

Mixtures of phenylalanine and phenylpyruvic acid exert more than an

additive effect in reversing the toxicity of thienylalanine. A relatively small amount of phenylpyruvic acid increases the ratio of thienylalanine to phenylalanine necessary for growth inhibition. Also, a small amount

TABLE I

*Reversal of Toxicity of Thienylalanine and Its Peptides by Phenylpyruvic acid*  
Test organism, *E. coli* 9723, incubated 17 hours at 37°

Phenylpyruvic acid*	Units, per 5 ml †	Supplements‡			
		TA§	TAG	GTA§	LTA
		Galvanometer readings			
<i>γ per 5 ml</i>					
5	0	47	57	49	57
5	0.3	49			
5	1	12	58	49	56
5	3	6	35	7	59
5	10		29		7
5	30		4		1
15	0	48	57	49	57
15	3	43		36	
15	10	26	46	17	56
15	30	12	44	8	3
15	100	2	39	4	
15	1,000		33		
50	0	48	56	48	57
50	1,000	40	53	47	56
50	3,000	36		46	
50	10,000	3	58	51	58

\* In the absence of exogenous phenylpyruvic acid, the concentrations inhibiting growth were TA, 1, TAG, 3, GTA, 1, LTA, 3 units per 5 ml

† 1 unit contains the equivalent of 1  $\gamma$  of  $\beta$ -2-thienyl-DL-alanine

‡ The abbreviations are as follows: TA,  $\beta$ -2-thienyl-DL-alanine, TAG,  $\beta$ -2-thienyl-DL-alanyl-glycine, GTA, glycyl- $\beta$ -2-thienyl-DL-alanine, LTA, L-leucyl- $\beta$ -2-thienyl-DL-alanine

§ Separate experiments

|| A measure of culture turbidity, distilled water reads 0, an opaque object 100

of phenylalanine allows phenylpyruvic acid to reverse the toxicity of thienylalanine in a competitive manner over a range of concentrations. Successively higher amounts of either phenylalanine or phenylpyruvic acid allow the other to exert greater reversing activities. At relatively high concentrations of phenylpyruvic acid, a low concentration of phenylalanine has no effect, presumably because transamination of thienylalanine and

TABLE II

*Effect of Combinations of Phenylpyruvic Acid and Phenylalanine  
on Toxicity of Thienylalanine*

Test organism, *E. coli* 9723, incubated 17 hours at 37°

$\beta$ 2 Thienyl- DL-alanine	DL Phenyl alanine	Supplements					
		Phenylpyruvic acid, $\gamma$ per 5 ml					
		0	5	10	20	50	100
		Galvanometer readings					
$\gamma$ per 5 ml	$\gamma$ per 5 ml						
0	0	71	72	69			
0.5	0	43					
1	0	3	69				
2	0		14	68			
5	0		9	42			
20	0			37	50		
50	0			22	50		
500	0			23	44	59	
1,000	0			9	43	57	64
2,000	0				38	54	
5,000	0				4	48	55
10,000	0					10	54
20,000	0						1
0	10	70	70	70	69	69	68
500	10	22	33	47	57		
1,000	10	1	22	32	49	63	67
2,000	10		4	15	34	56	65
5,000	10			3	1	49	60
10,000	10					2	49
20,000	10						1
0	20	69	70	69	76	71	69
500	20	49	55				
1,000	20	19	45	52	59		
2,000	20	4	7	22	42	61	
5,000	20		4	3	20	58	63
10,000	20				5	26	62
20,000	20					5	5
0	100	71		70			
2,000	100	68		68			
5,000	100	4		46			
10,000	100			4			

